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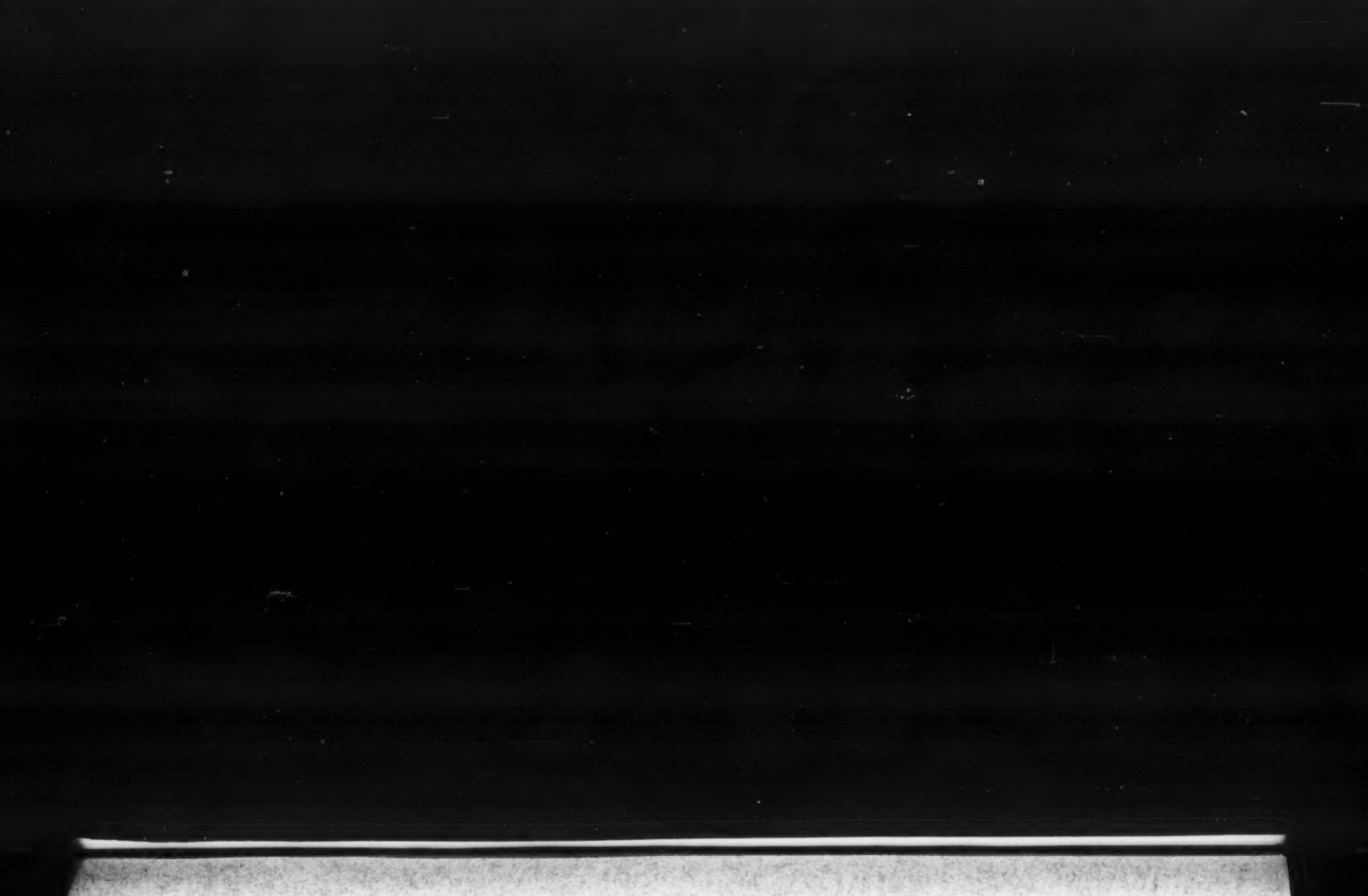

Vol. XLVIII *May, 1925* *No. 5*

BIOLOGICAL BULLETIN

THE FEEDING HABIT OF TERMITE CASTES AND ITS RELATION TO THEIR INTESTINAL FLAGELLATES.

L. R. CLEVELAND.[1]

More than 100 genera and approximately 1,200 species of termites are known; and each species is usually composed of five castes, with male and female individuals in each. Three of these castes, commonly referred to now as *first*, *second*, and *third forms*, are responsible for the reproduction of other individuals like themselves and for two other castes, *workers* and *soldiers*, which, although they possess reproductive organs, have given up the reproductive function (if they ever possessed it).

Nearly all the observations and experiments in the present paper have been carried out on one of the most common North American termites, *Reticulitermes flavipes* Kollar, whose castes (Thompson and Synder, '20) may be briefly described as follows:

(1) *First form*, which has three well-defined phases of development: (*a*) the nymphs (Figs. 1, 2), with long wing pads, creamy white body 1.3–1.4 mm. long, light brown eyes; (*b*) the winged adults, with long wings, dark brown body 6 mm. long, and black eyes; (*c*) the older males and females (Fig. 3), with enlarged abdomens and the scales of the shed wings, body 7–14 mm. long.

(2) *Second form*, which, like the first form, has three well-defined phases of development: (*a*) nymphs (Figs. 4, 5), with short wing pads and colorless body and eyes; (*b*) the young adults, with short scaly wing vestiges, straw-colored or grayish body 6–7 mm. long; (*c*) the older adults (Fig. 6), with wing vestiges, enlarged abdomen, body length 7–12 mm.

[1] Fellow (in the Biological Sciences) of the National Research Council, working at the Department of Medical Zoölogy, School of Hygiene and Public Health, Johns Hopkins University, Baltimore, Maryland.

(3) *Third form*, which also has three developmental phases: (*a*) the nymphs, wingless, with white head and body, and eyes that are invisible in the living or unstained specimen; (*b*) the young adults, no wing vestiges, head and body white, 7–9 mm. long.

(4) The *worker* (Fig. 7), wingless, with grayish abdomen, only two developmental phases, *i.e.*, nymphs and adults, salivary glands small and very little fatty tissue is present in the body, blind.

(5) The *soldier* (Figs. 8, 9), wingless, with elongated head covered with thick yellowish chitin, mandibles very large, long, dark brown, slender and curved, abdomen shorter than in other castes and more flattened, nymphs and adults only, no post adult growth as in reproductive castes.

Thompson ('17) showed that the newly hatched nymphs of *Reticulitermes flavipes*, 1.1 mm. long, although externally all alike, could be differentiated by their internal structures into two distinct types, namely, (*a*) reproductive nymphs, from which the three fertile adult castes develop, and (*b*) the worker-soldier nymphs, from which the sterile adult castes develop. By the time reproductive nymphs had attained a body length of 1.3–1.4 mm. they could be differentiated by their internal characters into nymphs of the first form and nymphs of the second form, which developed, finally, into the two respective reproductive adult castes. Soldier and worker nymphs could be differentiated internally by the time they had attained a body length of 3.75 mm. Nymphs of the third form could not be differentiated until a body length of 4 mm. was attained.

These five castes occur in most termites. Known exceptions are: the third form occurs in few, if any, species of the family Termitidae; a true worker caste is not present in the genera *Termopsis* Heer and *Neotermes* Holmgren, but a large-headed worker-like reproductive form is present; two genera, *Kalotermes* Hagen and *Cryptotermes* Banks, have no worker caste; the genus *Anoplotermes* F. Müller has no soldier caste; some species have as many as three types of soldiers, which, if counted as castes, make these species have seven castes, provided the five described above are all present.

Grassi ('93) calls the first forms "true" or "perfect" insects,

or "royal forms." The second and third forms he calls "substitute" and "complemental" forms, which forms, he thinks, are always ready to take the place of the royal forms in case of need. This author believes the castes are a product of environment and special feeding; what environment and special feeding, he does not consider. Bugnion ('12, '13) and Imms ('19) believe the castes are a product of the germplasm. The field observations that have been made (Snyder, '15, '16) support this contention. So do the morphological studies of Thompson ('17). But we really *know* absolutely nothing about what produces the castes. The question is badly in need of study. Jucci ('20),[1] who recently announced the discovery of a particular diet which brings about caste production, has added nothing but confusion, subtlely clothed in high-sounding phraseology, to the origin of a most interesting phenomenon.

From the description that was given of the castes, it may be clearly seen that very great morphological differences exist within a termite species, *i.e.*, the castes are quite distinct. It also seems likely that physiologically the castes are equally distinct.

The writer ('23*b*, '24*a*, '25*b*) has definitely shown that the removal of the intestinal protozoa from at least two genera of termites (*Reticulitermes* and *Termopsis*) makes it impossible for them to live on their normal diet of wood. He ('23*a*) has also shown that if termites harbor protozoa, they must feed on wood or cellulose. In the present paper a study of the various castes has been made in order to determine whether or not what is true for termites in general is also true for each caste throughout its life-cycle. In addition to this, some data on the physiological differentiation of castes and the relation of the castes to each other have been obtained.

Experiments and Observations.

Forty to fifty colonies of *Reticulitermes flavipes* were collected and have been kept for the past three years in the laboratory where they have been carefully observed. More than 300 second

[1] After this paper had gone to press a voluminous monograph by Jucci appeared, in which much attention is given to considerations of minor importance. A good beginning has been made, but the problem of the origin of termite castes has not been solved.

and third form young adults and 150 soldiers have been isolated from these colonies and have been used in experiments. And several hundred have been kept for observations. Thousands of workers and first forms have figured in experiments and observations.

One experiment, which was later duplicated five times with the same result each time, was carried out as follows: Five individuals of each of the five castes, workers, soldiers, third forms, second forms, and first forms, were placed in each of five large size sputum jars with food. These jars were kept in a moist chamber, so that the moisture, as well as temperature and light, would be identical in all the experiments. Experience has also shown that this is the best way to keep a colony of termites normal in the laboratory. Another experiment, which was duplicated three times with the same result each time, was carried out in the same manner as the experiment just mentioned except that the termites were kept in screw-top jars which were not placed in a moist chamber. In both of these experiments the second forms, the third forms, and the soldiers were all dead, in every instance, within three to four weeks, while the first forms and workers were able to live indefinitely.

Many observations were made daily throughout the course of these experiments and no castes except the workers and first forms were ever seen to take food. At various intervals several individuals were killed and their intestinal contents were carefully examined microscopically, with the result that no wood particles were ever present except in the workers, first forms, and soldiers—just how the soldiers came to have wood particles in their intestines and yet were not able to live will be made clear later. It seems quite evident, indeed, then, that the young second and third form adults do not feed on wood; that they get their nourishment perhaps in the form of salivary secretions from the xylophagous members of the colony.

Why do some castes die when placed by themselves? Is it because they do not take food? To test this point further, twenty experiments were carried out, ten where one worker was placed in a vial with two second form individuals and ten where one worker was placed in a vial with two soldiers. Under this condition, the second forms and soldiers were able to live in-

definitely. Thus one worker can at least support itself and two soldiers or two second forms. How much more a worker can do, was not determined.

Why do the second and third form young adults not take food when placed by themselves? A very large number of individuals of the second and third form castes were examined carefully for protozoa and in no instance were protozoa found after the final molt. In this connection it is interesting to note that every time any individual of any cast molts, its intestinal protozoa are lost, but in all molts, except the last one of the second and third forms, the protozoa are regained very quickly. Sometimes—though very rarely indeed—as the intestine slips off during the molting process, a portion of it is eaten before the protozoa die; but, as a rule, if an individual at the time of molting is placed to itself, it does not regain its intestinal protozoa and, as a consequence, dies within three weeks or thereabouts. But the first form regains its intestinal protozoa after the final molt. Why is it able to do this, while the second and third forms cannot? Obviously, one reason why the second and third forms cannot live by themselves after the final molt is because they have lost their protozoa. But this does not explain why they do not take food, for workers continue to feed after the protozoa have been removed from them experimentally.

Why and how are the protozoa lost? In an effort to throw light on this question, the second form was studied during the short period just before the final molt in which it can be distinguished externally from the first form. It was found that the protozoa gradually disappear during this period and that few, if any, are present at the time of the final molt. It must be noticed that there is also a dimunition in the protozoa in the first form during the period preceding the final molt, but not so great as in the second form, and the protozoa never disappear entirely. This progressive disappearance of the protozoa of the reproductive castes at this time is perhaps brought about by one or both of two things; namely, the salivary secretion which is taken during this period of rapid change and development destroys the protozoa and, as a consequence, wood-feeding must be given up; or, so much salivary secretion is taken that wood-feeding is thus made unnecessary and is, therefore, given up,

in which case the protozoa die due to wood starvation, just as happens experimentally (Cleveland, '25*b*) when any normal wood-feeding termite containing a large number of protozoa is starved. In all three reproductive castes it is quite evident that much less wood is eaten at this time; but in the first form the wood diet is not entirely given up; it may be curbed greatly, though never supplanted, by the salivary diet. It is interesting in this connection to note that the first forms eat much more wood shortly after the final molt and, because of this, the protozoa increase rapidly in number. They certainly receive no salivary secretion from workers for sometime if they leave (swarm) the parent colony to start a new colony, and nearly every one, if not every one, leaves or is killed. Their only food is wood until they rear workers to furnish them salivary secretions again, and when this is done, they again progressively cease to eat wood, finally giving up the habit entirely, at which time they lose all their protozoa and become dependent on the xylophagous members of the colony for the rest of their lives. It is interesting here to note that instinctively this dependence, which is perhaps inevitable, is well taken care of or looked forward to, because mostly workers are reared in the first brood of such a reproductive pair. But would they become dependent if not allowed to rear workers, that is if the larvæ were killed or taken from them?

We have already said that one reason why the second and third form young adults die when placed by themselves is because they lose their protozoa, and the protozoa are lost because these forms do not feed on wood, the second and perhaps more fundamental reason why death results when such individuals are isolated. But why do they not eat wood? According to Thompson and Snyder ('20) the jaw muscles and many others, particularly those in the head, of the first form degenerate during the post-adult stage. "This degeneration of the jaw muscles," they state, "is due to the fact that the reproductive forms are now fed by the workers on partly digested food and no longer masticate wood as they were compelled to do before the first broods of workers were raised." These authors also observed that the jaw muscles in the second and third forms in the post-adult stage had degenerated, though they did not state when the degeneration occurred. If, in the first forms, it occurs, as they

state, when workers supply them with "partly digested food," thus making the eating of wood unnecessary, may we not reasonably assume that in the second and third forms it also occurs when partly digested food is supplied them by workers and wood-feeding is permanently given up, that is at the time of the final molt or thereabouts, considerably earlier than in the first forms. If this is true, we know why the second and third form young adults when experimently placed by themselves do not eat wood and die; their jaw muscles have degenerated, thus making it impossible for them to eat wood, and they can only survive when fed by workers.

But what causes the jaw muscles to degenerate? Thomson and Snyder ('20) think it is due to disuse, brought about when the salivary diet takes the place of the wood diet. If this is true, then, the second and third forms must get more salivary secretion or partly digested food than the first form, since they lose the ability to eat wood and, of course, their protozoa that digest the wood for them, much earlier in life, at least two years earlier. If it is true that salivary secretion brings about a degeneration of the jaw muscles, why are the reproductive forms fed a salivary diet? Certainly not to make the jaw muscles degenerate, for this is surely only a consequence of some deeper, underlying reason for feeding a salivary diet to the reproductive forms. In other words, if the jaw muscles do not degenerate except through disuse, the salivary secretion is perhaps a necessity and may play a vital part either in accelerating or changing the course of development. On the other hand, if the jaw muscles degenerate in the absence of a salivary diet, that is not because of disuse, it may be that the function of such a diet is simply to take the place of a wood diet which becomes impossible. If this is true, the question, why do the jaw muscles degenerate, is perhaps as vital as caste production itself, which, if a result of food, would perhaps be stopped, or at least held in abeyance to some extent, if individuals were isolated very early. A few attempts have been made to get second forms early in the gradual decline of wood-feeding, which occurs simultaneously with a progressive dimunition in the number of protozoa. It was found that such second form individuals when isolated early can live by themselves longer than if allowed to remain with

workers for a while and isolated at a later stage of development, thus showing that at the time of the earliest isolation they had already begun to gradually lose the ability to maintain themselves and that they lose it more quickly when they remain with workers who fed them. Thus the decline in wood-feeding occurs anyway regardless of the time of the isolation, although more slowly seemingly when workers are not present. However, this experiment does not mean much, for long before the external differentiation has occurred which makes the distinction between the second and first form possible, several quite noticeably distinct internal microscopic differentiations had already occurred, thus showing that the differentiation cause had its origin much earlier and had been operating for sometime before the attempt to arrest it was made. Perhaps the workers could distinguish them and had been feeding them. To settle the question in this way, one should begin the isolation at an earlier stage. But these microscopic differences just referred to are only distinguishable in *Reticulitermes* after fixation and staining. Possibly in other genera the task will be less difficult, and we may be able to determine definitely what effect, if any, a salivary diet has on caste production, whether the decline in wood-feeding is caused by the salivary diet or whether the salivary diet has to be substituted for the wood diet after the jaw muscles have degenerated.

Since the second and third form young adults cannot live by themselves, it, of course, follows that they cannot start new colonies in the absence of workers. Incidently, the fact that they do not harbor protozoa shows beyond question, regardless of the fact that they cannot eat wood, that they never start new colonies in the absence of workers. This would be true just the same even if the protozoa were not absolutely necessary to their existence, because if new colonies were started by second and third forms, these colonies would not have protozoa in any of their castes. No such colonies have ever been found; consequently workers must be present when these forms head a colony.

It should be mentioned here that in the genus *Termopsis*, which has no true worker caste, it appears from the observations that have been made that the ability to eat wood is not lost in the second and third forms. But the observations on this genus are

really too few to warrant a conclusion further than that these forms certainly do not lose their protozoa and the ability to eat wood as early as they do in *Reticulitermes*, which has workers. If the degeneration of the jaw muscles occurs in the reproductive forms when and because workers supply them with a salivary or partly digested diet, we should not expect to find degenerate jaw muscles and enlarged dependent reproductive forms in those genera where workers are not present, unless, of course, the young nymphs play the same rôle that workers do in other genera.

We will now return to the soldier caste and take up the question, why are adult soldiers unable to live by themselves? Simply because their very large and heavily chitinized mandibles (Fig. 9) will not permit them to eat wood. They, too, like the second and third forms, lose the ability to eat wood but from a growth process rather than one of degeneration. Before their mandibles grew so large, they could eat wood and could live by themselves. Could these mandibles be altered by a change in diet? Did a diet produce them? We cannot answer either question.

Soldiers can digest wood, for they have protozoa in their intestines to do it for them, but are unable to eat it. They lose the ability to eat wood without losing their protozoa, for they get protozoa, just as all other castes do, very early in life from the ani of the xylophagous members of the colony. As we have said, owing to their very enormous and highly specialized mandibles (Fig. 9), they cannot chew wood, but they manage to ingest proctodael wood particles—partially digested perhaps—which have passed through the alimentary canal of those members in the colony capable of chewing wood. Their intestines are considerably smaller and they harbor a smaller quantity of protozoa than workers of the same size. Soldiers, then, are not as dependent, in one sense, on workers or permanent wood-chewing members of the colony as the second and third forms are, for they do not require totally predigested food, such as the salivary secretions upon which the second and third forms probably feed entirely after the final molt.

Summary.

All results were obtained from laboratory colonies which have been carefully studied during the past three years. Many of these results have been verified by field observations.

At every stage in the life-cycle of any caste where wood is eaten, protozoa are present. When wood is not eaten or obtained in some way, protozoa are never present.

Second and third form young adults have lost the ability to eat wood. The protozoa in these castes disappear concomitantly with the loss of the ability of their host to feed on wood, and by the time the wood-eating ability is lost, they have all disappeared. This occurs about the time of the final molt and is perhaps brought about by the feeding of salivary secretions which take the place of the wood diet. In all castes, the protozoa are lost during molting, but they are soon regained, except in the final molt of the second and third forms, in which forms they are never regained because, owing perhaps to the degeneration of their jaw muscles, these forms have lost the ability to eat wood. What causes the jaw muscles to degenerate is not definitely known. It may be inherent in these castes, as much a part of them as anything else. If it is, then the salivary-feeding is hereby made necessary and takes the place of the wood diet when the jaw muscles degenerate. But a more plausible possibility is that these forms are fed so much salivary secretion that they cease to feed on wood and because of this their jaw muscles degenerate through disuse, and thus the ability to feed on wood is lost forever.

The first form and the worker always eat wood, except in the post-adult stage of the life-cycle of the first form, where it, too, after having attained an old age loses the ability to eat wood and becomes dependent on the workers and young undifferentiated nymphs (when present) which it has reared. It is noteworthy that mostly workers are raised in the first brood.

Adult soldiers, owing to their large mandibles, cannot eat wood (cannot chew it), though they obtain it, together with protozoa from the ani of the xylophagous members of the colony. Soldiers, like workers, harbor protozoa throughout their life-cycle. Young soldiers (soldier nymphs), before they obtain the

large mandibles, can chew wood for themselves. So can the second and third forms, during early life.

A caste which cannot eat wood, or, thinking in terms of the protozoa, a caste which does not harbor protozoa, cannot live by itself. Such individuals are dependent on the wood-eating members of the colony for support; consequently adults of the second form, third form, and soldier castes must be supported by other members of the colony. But the soldiers, in one sense, are not as difficult to support as the second and third forms, since they can digest for themselves the partially digested woody material which has passed through the alimentary canal of the xylophagous members of the colony before they receive it; while the second and third forms, since they feed exclusively on the salivary secretions, must subsist entirely on predigested food.

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EXPLANATION OF PLATE 1.

FIGS. 1–9. The Castes of *Reticulitermes flavipes*.

FIG. 1. Nymph of first form, with long wing pads, creamy white body from 1.3–1.4 mm. long, light brown eyes.

FIG. 2. Side view of Fig. 1.

FIG. 3. Post adult first form queen, with enlarged abdomen, the scales of the shed wings, body length varies from 7–14 mm. This figure × 5.

FIG. 4. Nymph of second form, with short wing pads which never develop into wings, colorless body and eyes.

FIG. 5. Side view of Fig. 4.

FIG. 6. Post adult of second form queen, with wing vestiges, enlarged abdomen, body length varies from 7–12 mm. This figure × 5.5.

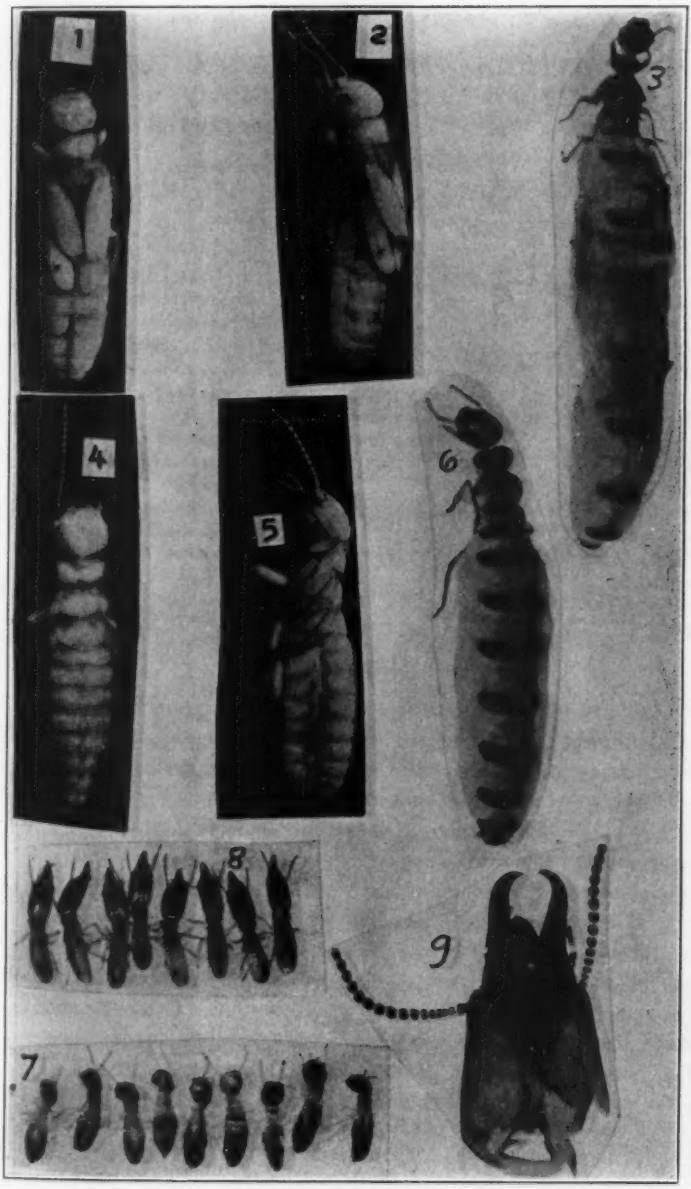
Note: The third form caste which for lack of space is not shown, has no wing vestiges, eyes cannot be seen except in stained material, is smaller having a body in the post adult stage of 7–9 mm.

FIG. 7. A group of workers enlarged three times.

FIG. 8. A group of soldiers enlarged three times.

FIG. 9. Mandibles of adult soldier which make the eating of wood impossible. × 16.

All figures (photographs) after Snyder.



L. R. CLEVELAND.

THE EFFECTS OF OXYGENATION AND STARVATION ON THE SYMBIOSIS BETWEEN THE TERMITE, *TERMOPSIS*, AND ITS INTESTINAL FLAGELLATES.

L. R. CLEVELAND.[1]

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INTRODUCTION.

In a previous paper ('24*a*) the writer reached the conclusion that xylophagous and protozoa-harboring termites are not able to live on their normal diet of wood after their intestinal protozoa are removed from them by incubation for 24 hours at 36° C. They die within three to four weeks if given a wood diet. When the protozoa are replaced, the termites concomitantly regain their ability to live indefinitely on a diet of wood or cellulose. Thus, the incubation, which removed the protozoa, did not kill the termites *per se*. Also, when termites from which the protozoa had been removed by incubation, were given a diet of fungus-digested cellulose, they were able to live indefinitely. Therefore, the ability to make use of cellulose (to maintain itself indefinitely on a wood diet an animal must be able to digest

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cellulose) seems to reside in the protozoa of the termites rather than in the termites themselves.

In the present paper the protozoa have been removed from the large Pacific Coast termite, *Termopsis*, by two other methods, namely, starvation, and oxygenation, with the same results in each case as were obtained by incubation; the termites were not injured although the ability to live on wood disappeared simultaneously with the loss of the protozoa.

In addition to this, the relation of each species of protozoa to its host and to its neighbors or fellow protozoa, has been worked out by employing the various protozoal-removing methods separately and by combining them. Differential defaunation or the removal of some species of protozoa without affecting the others was thus attained.

Termopsis AND ITS PROTOZOA.

Three species of *Termopsis* are known. Two of them, *T. angusticollis* Hagen and *T. nevadensis* Hagen, can be distinguished only by an examination of their winged adults. In many of the colonies studied, no winged adults have appeared; consequently it has been impossible to determine whether all the experiments were carried out on one or both of these species. The winged adults which appeared in two colonies were *T. nevadensis*. Both species occur in the same localities in Oregon, California and elsewhere on the Pacific Coast and probably harbor an identical protozoan fauna.[1] But for these reasons, it seems most feasible to avoid specific determinations in this paper.[1]

The protozoa of *Termopsis* were first described by Kofoid and Swezy ('19) as follows: "In *Termopsis angusticollis* four different species of protozoans are invariably present." These they described as *Trichonympha campanula* (Fig. 1), *Leidyopsis sphaerica* (Fig. 2), *Trichomitus termitidis* (Fig. 3), and *Streblomastix strix* (Fig. 4). They state that "in addition to these there are usually present minute forms of two, sometimes three species of flagellates," which these authors did not study. These same small forms have been observed by the writer, but they are very small indeed, and few in number, and have been considered of no moment in the experiments that have been carried out.

[1] Later observations show that both species were used and that they harbor the same protozoa.

With two[2] exceptions (*Leidyopsis* and *Trichomitus*), no special effort has been made to verify the morphological studies of Kofoid and Swezy. However, as will be seen later, the writer has had occasion to observe many of these protozoa in almost pure cultures, which simplifies the matter of morphological details considerably, for unless one has really seen this overwhelming mass of squirming, wriggling, undulating protozoa which greet the eye when a termite's intestinal content is viewed under the microscope, one cannot form any conception whatever of the immense difficulty envolved in attempting to study in detail any of them except the very large and dominant genus *Trichonympha*. Of course, if one has a suitable medium in which to dilute the intestinal contents, these difficulties of observation are obviated greatly, but Kofoid and Swezy state that they did not find such a medium; hence it was difficult for them to make out the finer structures of the two smaller forms, *Trichomitus* (?) and *Streblomastix*.[2]

Leidyopsis, when millions of individuals are viewed in a suitable medium and unobscured by other protozoa, is not nearly so rounded as Kofoid and Swezy have figures from stained specimens (Fig. 2). This rounding up which they show is an abnormality of fixation. In living material it occurs also unless the observation is made in a suitable medium (Cleveland, '25*d*).

In the *Termopsis* material—most of which came from Oregon, though three colonies were obtained from California—which the writer has studied there is certainly a species of *Trichomonas* present; whether or not *Trichomitus* is also present it seems almost impossible to say, though all evidence indicates that it is not. For instance, in those hosts which were experimentally freed of *Trichonympha* and *Leidyopsis*, thus affording a wonderful opportunity to study *Trichomonas* (and *Trichomitus* too if present), an opportunity which Kofoid and Swezy did not have, an axostyle (the distinction between *Trichomonas* and *Trichomitus*, a genus which Swezy founded in 1915, lies chiefly if not entirely in the presence of an axostyle in *Trichomonas* and the absence of it in *Trichomitus*) can be seen instantly in 50 per cent. of the individuals exclusive of *Streblomastix;* with more study the number of individuals in which an axostyle is visible increases

[2] *Streblomastix* has only four flagella. See addenda.

to 70–80 per cent.; with still more study after fixation and staining the percentage of individuals in which axostyles may be seen increases to 85–90 per cent., though never reaching 100 100 per cent. In other words, the number of individuals which at first glance would be diagnosed as *Trichomitus*, diminishes concomitantly with scrutiny of observation. Professor Kofoid when notified recently by letter of this finding and of the possibility that the species of *Termopsis* in California (although the protozoa in those colonies I examined were the same as in the Oregon *Termopsis*) might harbor *Trichomitus* while in Oregon they harbor only *Trichomonas*, wrote: "We find both *Trichomonas* and *Trichomitus* in the same hosts in California. We have regarded this *Trichomonas* as possibly the one described by Dogiel although we have not attempted to work it up." Professor Kofoid also kindly sent me slides which show an abundance of *Trichomonas*. However, in the papers of Kofoid and Swezy ('19), as noted by the quotations already given, no mention of the presence of *Trichomonas* appeared. This organism in *Termopsis* does not seem to me to be the same one which Dogiel [1] described as *Tetratrichomonas macrostoma* from *Rhinotermes* sp. of Uganda. The two hosts are widely separated and belong to two families; *Rhinotermes* Hagen belongs to the Rhinotermitidæ, and *Termopsis* Heer to the Kalotermitidæ. The species of *Trichomonas* in *Termopsis* has four anterior flagella (elsewhere the statement was made by the writer ('24*a*) that it had three anterior flagella, but more careful examination shows clearly that some individuals have four anterior flagella, which is perhaps the normal number), axostyle and undulating membrane, which place it definitely in the genus *Trichomonas*. I, therefore, describe it (Figs. 5, 6) as *Trichomonas termopsidis* sp. nov., found certainly in *Termopsis nevadensis* Hagen and probably in *T. angusticollis* Hagen.

This is not the time nor the place for a discussion of the genera *Trichomitus* and *Trichomonas*. If *Trichomitus* is present —though I am inclined somewhat to doubt it—it has behaved the same way as *Trichomonas* in the starvation and oxygenation experiments. But, since it is not certain that *Trichomitus* is present or even exists, no mention of this genus will be made in these experiments.

[1] Dogiel, V.; *Jour. russe de Zoöl.* (Petrograd), 1.

Experiments and Observations.

a. Starvation.

Ten experiments, employing altogether approximately five hundred termites, were carried out as follows: The termites were removed from wood and carefully freed of all wood particles which they attempted to cling to. Then they were placed in large Petri dishes which were kept in moist chambers. In this way the amount of moisture which previous work had shown most desirable was constantly supplied. All the way from one to fifty individuals were placed in a Petri dish during starvation, with the same results in all instances.

When starved in this manner, *Termopsis* begins to lose its large, dominant and principal wood-ingesting protozoön, *Trichonympha* (Fig. 1), by the end of the third day, and by the end of the fourth day perhaps half the individuals of this genus originally present are dead, although few, if any, of the other genera of protozoa have died. By the end of the fifth day, some termites have lost all their *Trichonympha*, while others still retain a few slowly moving, apparently weak individuals. By the end of the sixth day, no *Trichonympha* can be found in any termites, and perhaps half the individuals of the next largest protozoan, *Leidyopsis* (Fig. 2), have died. And in a few termites perhaps all or nearly all of *Leidyopsis* may be dead, but this is exceptional. Also, by this time a few individuals of the next smallest genus, *Trichomonas* (Figs. 5, 6), may have died, but not many. If starvation is continued through the seventh day, some termites completely lose their infection of *Leidyopsis*, while others harbor a few individuals until near the end of the eighth day. After eight days of starvation, then, the two large protozoa, *Trichonympha* and *Leidyopsis*, have all disappeared entirely. If the starvation is continued, *Trichomonas* now begins to die rapidly and by the end of the tenth day nearly all the individuals of this genus are dead, though perhaps one to two per cent. of the total number originally present live sometimes four or five days longer. *Trichomonas* struggles along and dies more slowly than *Trichonympha* and *Leidyopsis*. All of these protozoa feed on the wood particles which their host has eaten, but *Streblo-*

mastix (Fig. 4) does not. It may be dependent on the other protozoa or on the termites for its nourishment. It is difficult to say just when *Streblomastix* begins to die since this genus is much smaller than either of the other three and in the normal or not-starved termite is greatly obscured by the countless thousands of larger individuals, thus making it difficult to determine accurately the normal number present; but the number has perhaps diminished some by the end of the tenth day and by the fifteenth day a great many have died, though not all; some of them, in fact, live almost as long as the termites—three to four weeks.

During starvation most termites are active and appear normal for about fifteen days. However, as soon as many of the protozoa have died, after six days, say, it is not necessary to make a microscopical examination of the intestinal contents to determine what has happened, for there is now much more fluid than formerly present and it looks very muddy—the difference is quite characteristic and cannot be mistaken.

Why do termites, when not given food (wood), lose their protozoa? Do the protozoa die of actual starvation, and most of them much more quickly than their host? Three experiments were carried out which perhaps throw some light on this question. When termites are fed cellulose instead of wood for several months before being starved, and then are cellulose-starved, they lose their protozoa more slowly. For instance, it takes them at least one to two days longer to lose *Trichonympha*. The writer ('25*c*) has shown elsewhere that this termite (*Termopsis*) can live for more than a year and perhaps indefinitely in a perfectly normal manner on a diet of pure cellulose and may it not be true that when it is fed nothing but cellulose (wood is about 50 per cent.) for some time before being starved that it really has more food in its intestine for the protozoa when starvation is begun and for this reason the protozoa are able to live longer? If so, this indicates that when *Termopsis* is wood-starved that its intestinal protozoa, particularly *Trichonympha*, *Leidyopsis*, and *Trichomonas*, die of actual starvation long before their host. We should expect the protozoa to die first since they digest the wood for themselves and their host. And when they die, they perhaps give themselves as food to their host,

which is thus enabled to live considerably longer than its protozoa. In nature, termite protozoa may aid their host by giving themselves as food. It is not known how long the life-cycle of these protozoa is, but, if it is not longer than that of the parasitic protozoa that have been cultivated in artificial media from a single individual, countless millions of them must die daily in a single termite.

b. Oxygenation.

It has been fairly common knowledge for some time that oxygen in rather excessive or abnormal amounts is toxic for many, if not all, forms of animal life. Realizing there must be very little oxygen in the environment of intestinal protozoa, I concluded that they might for this reason be more sensitive to it than their host in an atmosphere of approximately 20 per cent. oxygen. Accordingly, it was decided to determine whether oxygen was more toxic for intestinal parasites than for their host. Obviously, for many reasons, termites are far superior to any other insect and perhaps any other animal for such a study. Workers, soldiers, and nymphs of the reproductive castes, can be counted on to have an infection of 100 per cent., approximately the same in all individuals of the same size and age. And there are millions of very large, active and highly specialized flagellates in a single insect. In fact, nearly half the body weight of the insect is made up of these protozoa. Where, then, could a better opportunity be found to study the effect of oxygen on intestinal flagellates? The termites are easily kept in the laboratory and will live almost indefinitely in tightly corked vials and flasks, for they can stand a very high percentage of CO_2.

These experiments were begun with a different end in view from the use that is made of them in this paper, for it was not thought that all the intestinal protozoa could be removed without injuring the termites too, and still less was the possibility contemplated that oxygen might entirely remove some genera of these flagellates before killing others. The other subject, that of the toxicity of oxygen proper, is being studied now and will be taken up in detail in a later paper.

It was found that if termites (*Termopsis*) were placed in fairly pure oxygen at one atmosphere pressure that all protozoa be-

longing to the genus *Trichomonas* (Figs. 5, 6) were killed within 24 hours and had disappeared from the termites' intestines, while *Trichonympha* and *Leidyopsis*, the two largest genera, and *Streblomastix*, the smallest genus, remained practically unaffected. Of course a few others were probably killed, but not many. The termites were not affected in the least by the oxygen. However, we should not expect them to be for many animals can live in an oxygen atmosphere at this pressure for a much longer period. The surprising thing is that the oxygen kills the protozoa so quickly and removes one genus completely long before the others.

When the termites were confined to the oxygen atmosphere for more than 24 hours, *Trichonympha*, *Leidyopsis* and *Streblomastix* began to die, though not all individuals of these genera were dead until about 72 hours. Sometimes they were dead a little earlier than this and sometimes a few hours later, the variation probably depending on the percentage of oxygen in the particular flask or vial containing them. The oxygen which was used was obtained by heating C. P. $K_2Mn_2O_8$ and was washed in NaOH before being run into the flasks and vials where it displaced most of the air. No effort was made to determine the percentage of oxygen in such an atmosphere, which must have varied considerably at times. Known percentages of oxygen at one and at more than one atmosphere pressure are now being used in the work on toxicity of oxygen for intestinal protozoa. The significant fact is that in all of more than 75 experiments which were carried out the protozoa were all removed in approximately 72 hours. In these 75 experiments, more than 1200 termites were used and none of them, in so far as could be determined by careful observation, ever suffered any ill effects from the oxygen. They easily live eight to ten days in an oxygen atmosphere which kills their intestinal protozoa in three days. No effort was made to determine just how long they would live, for after their intestinal protozoa have been taken from them, they cannot live more than three to four weeks in air.

c. Oxygenation and Starvation.

It was noticed in the starvation experiments that *Trichonympha* disappeared entirely after the termites were starved six days and that *Leidyopsis* had disappeared entirely by the

end of the eighth day of starvation. In the oxygenation experiments, *Trichomonas* disappeared entirely within 24 hours. It was obvious, then, that a combination of these two methods for removing the protozoa would yield interesting results. Accordingly, ten experiments, using approximately 400 termites, were carried out as follows: The termites were oxygenated, as in previous experiments, for 24 hours. This removed all protozoa belonging to the genus *Trichomonas*. About 100 of these termites were starved for six days, and 100 for eight days. In this manner, the first 100, or those individuals which were starved six days, were freed of *Trichonympha*, and the second 100, or those individuals which were starved eight days, were freed of *Trichonympha* and *Leidyopsis*. Thus, in those individuals that were starved eight days, *Streblomastix* only remained, while in those individuals that were starved six days, *Leidyopsis* and *Streblomastix* remained.

d. Wood-feeding after Intervals of Starvation and Oxygenation.

By starvation and by oxygenation and by a combination of starvation and oxygenation we have seen how it is possible to shift the protozoa about almost any way we wish. For instance, we can take out *Trichonympha* by starving six days and leave *Leidyopsis*, *Trichomonas* and *Streblomastix* uninjured; by starving eight days we can remove *Trichonympha* and *Leidyopsis*, leaving *Trichomonas* and *Streblomastix*, and then by oxygenating 24 hours we can remove *Trichomonas*, leaving only *Streblomastix*; or we may oxygenate first and remove *Trichomonas* which will leave *Trichonympha*, *Leidyopsis* and *Streblomastix*, and then if we starve these individuals for six days we have *Leidyopsis* and *Streblomastix* remaining; and by oxygenating for 72 hours all protozoa are removed. By this crisscross procedure we may obtain termites with five[1] protozoal combinations and protozoaless termites as follows: (1) *Leidyopsis*, *Trichomonas*, *Streblomastix;* (2) *Trichomonas*, *Streblomastix;* (3) *Streblomastix;* (4) *Trichonympha*, *Leidyopsis*, *Streblomastix;* (5) *Leidyopsis*, *Streblomastix;* and (6) no protozoa. Thus a wonderful opportunity is afforded for studying the relation of each of the four genera of

[1] Two more combinations have been obtained recently. See addenda for a tabulation of all combinations.

protozoa to its host and to its neighbors or fellow protozoa, for none of these six groups of termites was injured in the least by the methods employed in removing the protozoa. Each group would feed on wood just as it did before the protozoal alterations were made.

Each of these six groups was now fed wood and kept to itself in the same environment of temperature, moisture, and light. Each group contained about fifty individuals. Controls, or termites that had not been treated in any way, were also kept with these five groups. The results of feeding wood to each group may be briefly stated as follows:

(1) *Termites with Leidyopsis, Trichomonas and Streblomastix.*—In normal termites in nature, *Trichonympha* for some reason is perhaps 1000 times as numerous as *Leidyopsis*, and this ratio is fairly constant, although we do not know what makes it so. *Trichonympha* is the dominant genus in size at least and probably in number too—*Trichomonas* and *Streblomastix* may sometimes be as numerous as *Trichonympha*, but they are much smaller. A most interesting thing happens to *Leidyopsis* when its dominant neighbor, *Trichonympha*, is killed; it multiplies rapidly, soon increases very greatly, indeed, in number, and in 20–30 days has filled up the space made vacant in the termite's intestine by the removal of *Trichonympha*. This condition remains permanently and the group of termites is able to live indefinitely on a wood diet. *Leidyopsis*, then, is not only able to take the place of the dominant *Trichonympha* in number but can also take its place as the chief symbiont. As we shall see when we come to study the group of termites with *Trichomonas* and *Streblomastix*, or the group with *Streblomastix*, *Trichonympha*, in nature, is by far the most important symbiont. We know this, even though we were not able to get a pure culture of this genus, because *Leidyopsis* is present in too small a number to be of much importance and *Trichomonas*, as group (3) shows, is not of very great moment as a symbiont. So, in nature, *Trichonympha* is of most value to termites, because for some reason it is dominant over *Leidyopsis*; although, under experimental conditions, *Leidyopsis* can become of as great value to its host as *Trichonympha* is in nature.

(2) *Termites with Trichomonas and Streblomastix.*—When

Leidyopsis, although it is usually never present in nature in nearly such large numbers as *Trichomonas* and *Streblomastix*, is removed the same thing happens to *Trichomonas* that happens to *Leidyopsis* when *Trichonympha* is removed, namely, *Trichomonas* multiplies rapidly and increases very greatly in number for about 30 days, but it never completely fills the intestine of its host. These termites are active for approximately 60–70 days on the average, but after this period many of them die, although some of them live considerably longer and a very small percentage may be able to live indefinitely. However, under the present conditions, we may conclude that the symbiosis between *Termopsis* and its intestinal protozoa is very greatly damaged by the removal of *Trichonympha* and *Leidyopsis*. *Trichomonas*, under these experimental conditions, is undoubtedly of some value to its host, though certainly not as much as either *Trichonympha* or *Leidyopsis*. It is able to keep its host alive for at least 40–50 days, for when it is removed, as we shall see in group (3), the termites die 40–50 days earlier. It is possible that *Trichomonas* in this case has to support *Streblomastix*, as well as its host, for *Streblomastix* cannot live alone as group (3) shows. *Streblomastix* now, as was not the case when only *Trichonympha* had been removed, increases in numbers considerably, and, if a method for removing it without removing *Trichomonas* at the same time were available, *Trichomonas* might then be able to keep practically all of its hosts alive indefinitely. This, however, being a possibility which has not been tested, more work must be done before we can speak with exactness upon the precise ability of *Trichomonas* to keep its host alive.

(3) *Termites with Streblomastix.*—*Streblomastix* does not increase in number; on the contrary, it gradually diminishes, and its host dies within three to four weeks, just as when all protozoa are removed. We conclude, then, that *Streblomastix* is not a symbiont, for it does not seem to be of any value to its host. Incidently, this protozoön, unlike the other three genera, does not ingest wood particles from the intestine of its host, which also suggests that it plays no part in digesting food for its host. *Streblomastix* may depend on the other protozoa for its support.

(4) *Termites with Trichonympha, Leidyopsis, and Streblomastix.*—These termites are able to live indefinitely and it is

not possible to note any change in their protozoa. Perhaps *Trichonympha* takes the place of *Trichomonas*, but if this occurs, it cannot be detected because there are so many *Trichonympha* anyway. The loss of *Trichomonas* is of no consequence.

(5) *Termites with Leidyopsis and Streblomastix.*—*Leidyopsis* multiplies in this group as in group (1), and perhaps takes the place of *Trichomonas* as it does of *Trichonympha*. These termites, as those in group (1), are able to live indefinitely. Since *Streblomastix*, as group (3) shows, is of no value to its host, we may conclude that *Leidyopsis* alone, without the modicum of assistance from *Trichomonas* such as it received in group (1), is able to keep it shost alive indefinitely.

(6) *Termites with No Protozoa.*—These termites eat wood just as those in the other five groups, but are not able to live longer than three to four weeks. The inability to maintain themselves on their normal diet of wood is caused by the removal of the intestinal flagellates, particularly *Trichonympha* and *Leidyopsis*, from them.

Discussion.

Intestinal protozoa must live in an environment with a smaller percentage of oxygen than their hosts, and should, therefore, experience the greater difficulty when the oxygen environment of the host is raised from 20 to 100 per cent., provided, of course, the oxygen percentage of the parasite's environment does not increase correspondingly with that of its host. For instance, if there is normally, say, 1 per cent. of oxygen in the parasite's environment and 20 per cent. in that of the host, when the host is placed in an atmosphere of 100 per cent., the percentage of the host's environment is thus increased only five times, while that of the parasite is increased eighty times. On the other hand, if the oxygen percentage of the parasite's environment increases correspondingly to that of its host, or, as in this case, five times, then the parasite would be in 5 per cent. oxygen when its host was in 100. When termites are placed in 100 per cent. oxygen, the oxygen percentage of their parasite's environment may be increased much more than their own, and the parasites are killed, just as any animal would be with so great an oxygen increase. If this is true, the parasites can undergo as great change in

oxygen as their host, and oxygen is really not any more toxic for them than for their host. They die, then, while their host is uninjured, because the oxygen percentage of their environment increases many times more than that of their host.

In larger animals with a different system of respiration, the oxygen percentage of host and parasite may increase correspondingly, in which case it would be impossible to kill the parasites of, say, a vertebrate by confining it in an oxygen atmosphere without at the same time killing the vertebrate, unless, of course, oxygen is actually more toxic for the parasites. At any rate, other parasites may be killed by the use of oxygen if we can develop a method of getting it to them.

When termites are starved, their largest protozoa die first, but when they are oxygenated, their next to the largest one dies first. What is the cause of this? The larger and more active ones may require more food than the smaller ones, and for this reason starve more quickly, if starvation is the actual cause of death. Or it may be that the smaller ones are partly nourished by their host or by their larger protozoan neighbors. In the case of oxygenation, the smaller ones may die first because of the higher ratio of surface-volume exposure which they have.

One interesting problem which this study brings out is, what maintains the fairly definite ratio between the four genera of protozoa? The host may produce a reaction product for each genus which inhibits its multiplication beyond a certain point. However, this does not seem very likely, for when *Trichonympha* is removed, *Leidyopsis* takes its place, and when *Leidyopsis* is removed, *Trichomonas* partly takes its place. The protozoa may inhibit the reproduction of each other beyond a certain point. And another possibility is the question of struggle for food which must go on where such a large number of protozoa are present.

Summary and Conclusions.

The termite which was used in these experiments belongs to the genus *Termopsis*. Very probably two species, *T. nevadensis* Hagen and *T. angusticollis* Hagen, have been used. These two species are so nearly alike that they can be distinguished at present only by a study of their winged adults, which were not

present in most of the material used. The two species probably harbor an identical protozoan fauna.

Four genera of protozoa are invariably present in these termites. These in order of size are: *Trichonympha* (Fig. 1), *Leidyopsis* (Fig. 2), *Trichomonas* (Figs. 5, 6), and *Streblomastix* (Fig. 4). Kofoid and Swezy ('19) claim that another genus, *Trichomitus* (Fig. 3), is also present, but, for reasons given in this paper, it is impossible to say whether or not *Trichomitus* is present. If it is, it reacted in every way as *Trichomonas* did, and has the same relation to its host and fellow protozoa as *Trichomonas*.

Two methods, starvation and oxygenation, for removing the protozoa are given. By using each method separately and by a combination of the two it was possible to get five different combinations of the protozoa, without injuring the termites in the least. By starving for 6 days, *Trichonympha* was removed entirely; by starving for 8 days, *Leidyopsis* was removed completely; by oxygenating for 25 hours, *Trichomonas* was entirely removed; by oxygenating for 24 hours and starving for 6 days, *Trichomonas* and *Trichonympha* were removed; by oxygenating for 24 hours and starving for 8 days, *Trichomonas*, *Trichonympha* and *Leidyopsis* were removed; by oxygenating for 72 hours, all protozoa were removed. From this we get one group of termites with no protozoa in them and five groups with a different combination of protozoa in each group as follows: (1) *Leidyopsis*, *Trichomonas*, *Streblomastix;* (2) *Trichomonas*, *Streblomastix;* (3) *Streblomastix;* (4) *Trichonympha*, *Leidyopsis*, *Streblomastix;* (5) *Leidyopsis*, *Streblomastix;* and (6) no protozoa. By feeding the normal diet of wood to each of these groups of termites it was possible to work out the relation of each protozoan genus to its host and to its neighbors or fellow protozoa.

When group (1), which contained *Leidyopsis*, *Trichomonas* and *Streblomastix*, was fed wood, *Leidyopsis* multiplied rapidly, increased greatly in number and was able in 20–30 days to fill the vacant space made in the host's intestine when *Trichonympha* was removed. This group is able to live indefinitely.

When group (2), which contained *Trichomonas* and *Streblomastix*, was fed wood, *Trichomonas*, like *Leidyopsis* in group (1), multiplied rapidly and increased greatly in number for about 30

days, but was never able to fill the intestine with protozoa, that is to say, *Trichomonas* was never able to entirely take the place of *Trichonympha* and *Leidyopsis* in volume. Most of the termites of this group were able to live 70–80 days and some of them longer, although very few, if any, were able to live indefinitely. *Trichomonas*, then, is of some value to its host as a symbiont. It can keep its host alive 40–50 days longer than the host would be able to live without it. If *Streblomastix*, which is certainly not of any value to its host and may have to be supported by *Trichomonas* in this group, were not present, *Trichomonas* might be of more value to its host.

When group (3), which contained *Streblomastix*, was fed wood, death resulted within three to four weeks, the same time it occurs when all protozoa are removed. *Streblomastix* did not multiply at all; on the contrary, it gradually diminished in number. This protozoön, then, is not a symbiont. It may either receive its nourishment from its host or from the other protozoa directly, probably the latter.

When group (4), which contained *Trichonympha*, *Leidyopsis* and *Streblomastix*, was fed wood, all individuals were able to live indefinitely. The removal of *Trichomonas* did not seem to affect the symbiosis at all. *Trichonympha* perhaps took the place of *Trichomonas* very quickly.

When group (5), which contained *Leidyopsis* and *Streblomastix*, was fed wood, *Leidyopsis* multiplied and increased in numbers just as it did in group (1), perhaps taking the place of *Trichomonas*—though this could not actually be seen—just as it did that of *Trichonympha*. These termites were able to live indefinitely. *Leidyopsis*, even though in nature its ratio to *Trichonympha* is approximately 1 : 1000, can take the place of *Trichonympha* under experimental conditions both in number and in ability to keep its host alive indefinitely. In nature, however, *Trichonympha* must be the chief symbiont, for it is so much more numerous than its closest neighbor, *Leidyopsis*.

When group (6), which contained no protozoa, was fed wood, death resulted within three to four weeks.

The results of these experiments, as regards the symbiotic relationship between termites and their intestinal protozoa, are in accord with those obtained by the incubation method (Cleve-

land, '24*a*). The symbiosis between these insects and their intestinal flagellates has now been clearly demonstrated by three entirely different methods, namely, incubation, starvation, and oxygenation.

ADDENDA.

Since this paper went to press sometime ago several additional observations of interest have been made.

Thirteen colonies of *Termopsis nevadensis* and six colonies of *Termopsis angusticollis* have been obtained. The protozoa in all of these are the same.

By dry fixation and staining with Wright's stain one and three minutes respectively it has been possible to demonstrate beyond question four anterior flagella on *Trichomonas*. No organisms with three anterior flagella, the number *Trichomitus* is said to possess, have been observed. Hence it is quite probable that *Trichomitus* does not occur in *Termopsis*. Another interesting result was obtained by this method of fixation and staining: It was discovered that *Streblomastix* has only four flagella, instead of six, the number figured by Kofoid and Swezy ('19). The flagella stand out as big red lines which may be counted almost as easily as so many red pencils. Perhaps this method of fixing and staining will prove valuable in determining the flagella number of other protozoa.

By oxygenating *Termopsis* at a pressure of 1.5 atmospheres for 7 hours it has been possible to remove both *Trichomonas* and *Streblomastix* from many, though never all, hosts without seriously effecting *Trichonympha* and *Leidyopsis*. When termites oxygenated in this way are starved for six days, *Trichonympha* disappears entirely, thus making it possible to obtain termites with only *Leidyopsis*. This gives two more protozoal combinations, in addition to the five already obtained, *viz.* termites with *Trichonympha* and *Leidyopsis*, and termites with *Leidyopsis*. It now seems desirable to tabulate all the protozoal combinations; show how they were obtained, and how, if at all, they effect their hosts.

TABLE I.

RESULTS OF VARIOUS METHODS WHICH HAVE BEEN EMPLOYED IN REMOVING ONE OR MORE GENERA OF PROTOZOA FROM THE LARGE PACIFIC COAST TERMITE, *Termopsis nevadensis* Hagen.

Every host in nature always harbors each genus. − = absent, *i.e.*, treatment killed all protozoa of this genus and + = present, *i.e.*, treatment had no effect.

Methods of Treatment.	The Protozoa.				Result of Treatment on Host.
	Trichonympha.	*Leidyopsis.*	*Trichomonas.*	*Streblomastix.*	
1. Starvation for 6 days	−	+	+	+	Lives indefinitely.
2. Starvation for 8 days	−	−	+	+	Lives about 10 weeks.
3. Oxygenation for 24 hours at 1 atm.	+	+	−	+	Lives indefinitely.
4. Oxygenation for 24 hrs. at 1 atm. Starvation for 6 days	−	+	−	+	Lives indefinitely.
5. Oxygenation for 24 hrs. at 1 atm. Starvation for 8 days	−	−	−	+	Lives 3–4 weeks.
6. Oxygenation for 7 hrs. at 1.5 atms.	+	+	−	−	Lives indefinitely.
7. Oxygenation for 7 hrs. at 1.5 atms. Starvation for 6 days	−	+	−	−	Lives indefinitely.
8–13. Oxygenation, 1 atm., 72 hrs., 1.5, 9 hrs., 2, 5 hrs., 2.5, 2 hrs., 3, 1 hr. and 5 min., 3.5 40 min.	−	−	−	−	Lives 3–4 weeks.

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EXPLANATION OF PLATE 1.

The Protozoa of *Termopsis*. Figs. 1–4 after Kofoid and Swezy; Figs. 5, 6 original.

FIG. 1. *Trichonympha campanula*. × 300.

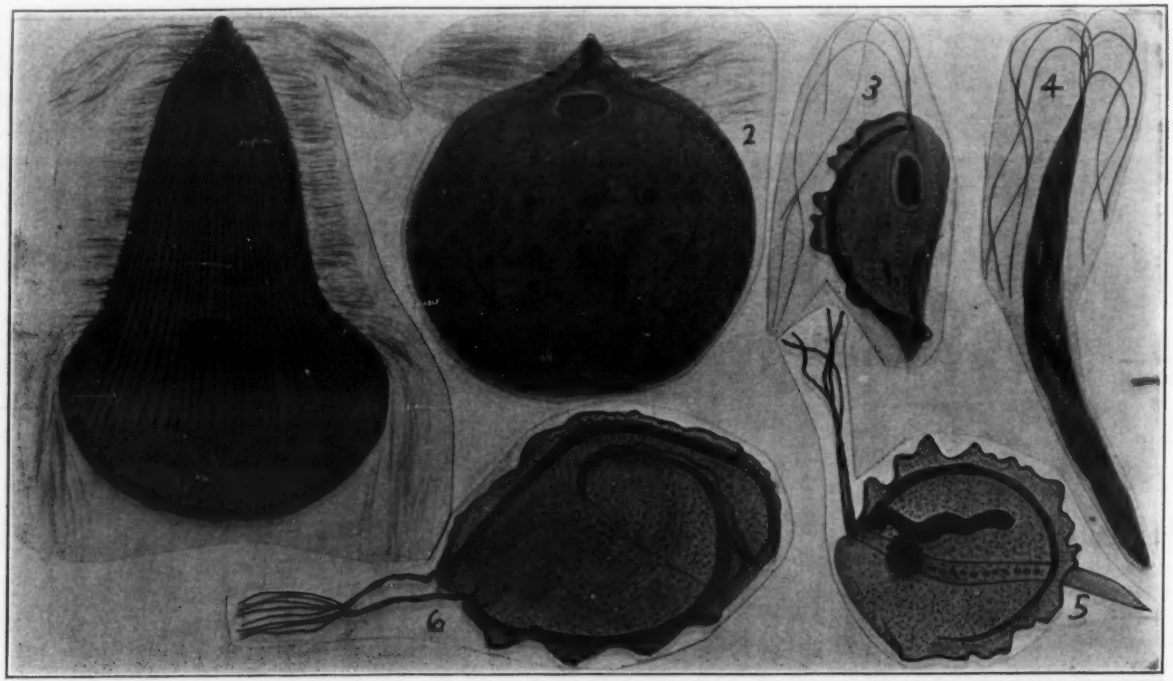
FIG. 2. *Leidyopsis sphaerica*. × 300.

FIG. 3. *Trichomitus termitidis* which may be a synonymn of *Trichomonas termopsidis* (Figs. 5, 6). × 625.

FIG. 4. *Streblomastix strix*. × 2500.

FIG. 5. *Trichomonas termopsidis* fixed in weak Flemming's fluid. Note parabasal body which is not present in Fig. 6 because this organism was fixed in Schaudinn's fluid which nearly always makes the parabasal invisible. × 1440.

FIG. 6. *Trichomonas termopsidis* dividing form fixed in Schaudinn's fluid. Paradesmose has just disappeared. Note doubling of nuclei, axostyles, anterior flagella, undulating membrane, and chromatic basal rod of undulating membrane. Parabasal body does not appear because of fixation in Schaudinn's fluid. × 1440.



L. R. CLEVELAND.

SOME PHYSIOLOGICAL DISTINCTIONS BETWEEN FRESHWATER AND MARINE ORGANISMS.

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I.

Aquatic organisms are in general distinctly divided into those species inhabiting fresh water and those inhabiting sea water. Yet this division is evidently not a phylogenetic one, since closely related species live in either (Quinton, '04). Only in a few species are the same individuals capable of passing from one medium to the other. The transition zones of brackish water are relatively small in extent and very variable, still there are a few organisms which thrive only in such diluted sea water.

Particularly the distribution of fishes between fresh water and sea water has excited the interest of biologists. Paul Bert tried to find why fish died when taken from their usual medium and placed in the other ('71, '73). For marine animals he ('83) reached one important conclusion, namely, that the toxic effect was not always due to the dilution of the substances dissolved in sea water. This conclusion has been thoroughly confirmed by the subsequent observations of Loeb ('03) and others.

Practically no further analysis of this problem could be made without careful quantitative studies. Such were undertaken by Sumner ('06) upon marine teleost fishes. He found significant changes in the water content and in the salt content of the animals during their immersion in diluted sea water. Thus the deleterious effect seemed to be a loss of essential body constituents, because the animals were unable to regulate favorably their integumental permeability under the new circumstances.

Meanwhile a new line of attack came to light with the discovery of salt antagonisms by Ringer ('82). Loeb ('00) found that marine organisms were readily affected by the disturbances of the balance between salts, but were almost independent of osmotic pressure changes. Thus ('03), the marine crustacean *Gammarus* endured dilution of the seawater medium just as well

if the diluting fluid was distilled water as if it was a sugar solution isotonic with sea water.

It is important to realize that various groups of animals differ greatly in their ability to control the interchange of substances between body fluids and external environment (Semper, '80). In all metazoa the covering epithelial layer of the body carries on an active regulation of the interchanges, so that the outer medium affects the more vital tissues only after it has with surprising slowness changed the composition of the internal medium (Adolph and Adolph, '25). Thus in all these animals there are two lines of defense, two regulating surfaces which must be penetrated. To illustrate several grades of this ability to control the interchanges at the body surface, we may cite the mammals, in which it is well known that the skin is impermeable at all times; the aquatic amphibia, in which the permeability is controlled in such a way that significant chemical interchanges between body and environment are strongly opposed; and the annelid worms, in which the interchanges are still less selected. In the teleost fishes essential interchanges are limited to the gill surfaces (Sumner, '06). In elasmobranch fishes the entire skin is partially permeable, while in invertebrates the body fluid often interchanges freely with the medium.

We have attempted to compare the vital resistance to the penetration of chemical agents in freshwater animals and marine animals. For this purpose we have chosen those animals whose entire body surface is normally permeable in some degree, namely, small invertebrates. In them the surface mass ratio is sufficiently large that effects of the chemical environment quickly manifest themselves.

II.

An excellent measure of the amount of intègumentary regulation performed by organisms is the rate of change of body weight when the organisms are transferred from their usual medium to a solution of different chemical composition (Adolph and Adolph, '25). Thus, when freshwater animals are immersed in salt solutions there is a rather sudden loss of water, so that the body volume quickly reaches a new lower level. Seawater animals placed in diluted sea water gain in volume, even though the

dilution be carried out with an isotonic solution of a non-electrolyte. Several species of invertebrates, both freshwater and marine, have been compared in this manner. The flatworm *Dendrocælum* and the earthworm *Lumbricus*, both of which inhabit fresh water, reach the new volume level in about 5 hours. The marine annelid *Phascolosoma*, when placed in sea water diluted to half its normal concentration, attained the new volume level in less than 2 hours. Evidently *Phascolosoma* adjusts to the new environment more rapidly than the freshwater species; it has less resistance to the penetration of water through its surface.

We wished to know whether dissolved substances likewise penetrated at different rates through the body coverings of these two classes of animals. After the first adjustment to the immersion in salt solution by loss of water, freshwater animals remained at the new level of volume for several days, and when replaced in fresh water recovered two thirds of the original loss. *Phascolosoma*, however, in diluted sea water lost slowly a considerable amount of the dissolved substance of the body fluids, so that when finally returned to normal sea water very little water passed from the body compared to that originally gained.

A still larger number of animals we have compared with respect to their resistance to solutions of salts and other substances. A list of these species, and the highest concentrations of certain solutes which they can just survive for a considerable length of time, are given in Table I. An important conclusion can immediately be drawn from these data; namely, that for freshwater organisms the osmotic pressure of the medium usually limits survival, while for marine organisms a great range of concentrations can be resisted. Thus, marine Gammarus will live indefinitely if transferred to sea water diluted with distilled water up to 0.5 per cent. (0.005 M), or concentrated by the addition of salts up to 160 per cent. (1.56 M, corrected for ionization). Freshwater *Gammarus*, on the other hand, are usually killed by immersion in any solution of a concentration equivalent to 0.35 M. Gradual dilution or concentration of the medium does not appreciably extend these limits.

The general significance of the conclusions from all the above experiments we interpret to be that both dissolved substances

TABLE I.

MAXIMUM SURVIVAL CONCENTRATION OR PERCENTAGE OF VARIOUS DISSOLVED SUBSTANCES WHICH WERE ENDURED BY 3 FRESHWATER SPECIES AND 3 MARINE SPECIES AT 22° C. FOR THE ARBITRARY TIMES SPECIFIED.

Figures in parenthesis are molar concentrations, corrected for ionization.

Solutions in Distilled Water.	Sea Water.	Urea, M.	Glycerol, M.	Glucose, M.	Sucrose, M.	NaCl, M.	KCl, M.	$CaCl_2$, M.	Ringer,[1] M.	$MgCl_2$, M.
Gammarus fasciatus (Crustacean) 5 hrs.	100% (0.97)	0.35	0.35	0.38	0.30	0.20 (0.35)	0.04 (0.07)	0.03 (0.08)	0.26 (0.43)	0.14 (0.35)
Phagocata gracilis (Flatworm) 24 hrs.	35% (0.33)	0.32	0.32	0.32	0.23	0.16 (0.28)	0.04 (0.07)	0.04 (0.11)	0.15 (0.26)	0.10 (0.25)
Paramecium caudatum (Protozoan) 1 hr.	17% (0.16)	0.32	0.25	0.20	0.20	0.11 (0.20)	0.07 (0.13)	0.05 (0.14)	0.11 (0.20)	0.12 (0.30)
Isotonic Solutions Added to Sea Water.	Distilled Water, %.	0.88 M Urea, %.	0.88 M Glycerol, %.	0.88 M Glucose, %.	0.88 M Sucrose, %.	0.54 M NaCl, %.	0.54 M KCl, %.	0.30 M $CaCl_2$, %.		
Gammarus locusta (Crustacean) 5 hrs.	99½	70	99½	65	85	80	5	21		
Procerodes wheatlandi (Flatworm) 24 hrs.	98	70	97	90	95	50	4	40		
Copepod (Crustacean) 1 hr.	60	60	60	50	60	60	3	15		

[1] Unbuffered Ringer's solution was made by mixing 97 volumes of 0.54 M NaCl, 2 volumes of 0.54 M KCl, and 1 volume of 0.30 M $CaCl_2$. This was taken to be 0.54 M.

and water penetrate the tissues of marine animals faster than they pass through the covering layers of freshwater animals. But in both cases the presence of salts in abnormal proportions in the medium renders the permeability of the integument high.

The greater permeability to salts and water in marine organisms accords with the ordinary conditions of existence for these animals. Marine animals are constantly bathed in a medium which is physiologically as suitable in inorganic composition as any internal one (Fredericq, '22). Freshwater animals, on the other hand, cannot afford to lose dissolved substances from their bodies nor to allow the entrance of water into their bodies up to the point where osmotic equilibrium would result.

Osmotic pressure changes *per se* are evidently not particularly deleterious to the vitality of internal tissues, providing the integument does not attempt to regulate against them. In marine organisms the freer penetration of solutes allows the internal medium to keep pace with the outer medium as regards composition, and so long as the salt balance is preserved, no essential functions are inhibited.

Morphologists have often attributed the chemical resistance of freshwater organisms to their possession of an outer cuticulum. Perhaps the resistance referred to is the resistance to the penetration of protein-precipitating agents, and in this a proteinaceous cuticulum probably assists. But under ordinary conditions the cuticulum is by no means either impermeable or semipermeable. Rather the living integument is responsible for the maintenance of restricted or selective permeability.

III.

A second significant characteristic of freshwater animals appears to be that their body fluids have a lower osmotic pressure than those of marine organisms.

Whether there is a relation between toxicity and tonicity we have attempted to investigate by measuring the survival of plasmolyzed *Spirogyra* filaments. With a variety of plasmolysing agents it was found that the toxic concentration was almost exactly the lowest one which produced distinct permanent plasmolysis. By gradually increasing the concentration of the medium, both toxicity and plasmolysis were prevented. Now, in

all the freshwater animals studied, toxic effects followed immersion in a concentration of about 0.35 M non-electrolyte solutions, or the equivalent concentration of electrolytes. Fredericq ('98) and Botazzi ('08) have shown that the tissue fluids of freshwater animals always have osmotic pressures less than half of that of sea water ($\Delta = 0.8°$ C.), and usually have only one eighth to one fourth of that of sea water; while marine animals have body fluids which are exactly isotonic with sea water. We can thus probably regard the maximum survival concentration for freshwater animals as a measure of the osmotic pressure of their body fluids. All the freshwater animals studied evidently had, therefore, internal osmotic pressures equivalent to 0.20 to 0.35 M.

The acclimatization of marine organisms to changed osmotic conditions contrasts to that for freshwater organisms. In several instances marine animals placed in glass-distilled water died, yet most of them survived a mixture of 98 or 99 per cent. of distilled water with only 2 or 1 per cent. of sea water. In other words, most marine animals are able to live after abrupt change to almost pure fresh water, providing that the remaining salts are present in physiological proportions. Gradual dilution of the sea water over several days did not materially help marine animals to endure pure water, though complete acclimatization has been secured over long periods of time by other observers (Beudant, '16, Plateau, '71, Semper, '80).

For freshwater organisms, gradual increase in the concentration of the medium did not greatly increase the maximum survival concentration. It is evident from this that plasmolytic effects are not the important ones in producing this toxicity. In diluted sea water all the freshwater animals studied except *Gammarus* were killed in concentrations less than half of that of the seawater at Woods Hole ($\Delta = 1.81°$ C.), and acclimatization never increased this toxic limit up to half of the concentration of the sea water. Similar acclimatizations to specifically poisonous substances have been demonstrated by Davenport and Neal ('96) and numerous other investigators, so that it seems doubly certain that the ultimate toxic effect is not plasmolytic. Moreover, the toxic effect cannot be attributed to sudden volume or concentration changes such as are brought about by diffusion. Certain balanced solutions such as sea water can be resisted in

spite of their high concentration by freshwater *Gammarus*; there is no interference with the regulatory activity of the integument. At this point we approach the problem of the conditions for the survival of the internal tissues, which are evidently very different from the conditions which the external medium may impose when an integument is interposed.

IV.

It appears, therefore, that freshwater organisms are strongly contrasted to marine organisms with respect to their ability to adjust to changes in their chemical environment. Marine organisms in general maintain a higher degree of interchange of inorganic materials with their surroundings. Freshwater organisms, on the other hand, have a more restricted and selective interchange, and thus dissolved materials are kept inside their bodies and water is kept outside. In protecting themselves from their environment through the retention of salts, freshwater organisms have laid themselves open, paradoxically, to osmotic disturbances of the integument. Marine organisms, by allowing the environing medium to serve as the physiological fluid, are able to endure a much greater change in the environing medium, than are freshwater organisms with their greater regulation of the internal medium. This greater change is one of concentration only, however, for both groups of animals are equally susceptible to variations in the proportions of salts in the medium. This condition in the freshwater animals is entirely due to the peculiar and variable type of permeability found in the integuments of this group, whereby they normally maintain a concentration difference between outside and inside fluids, as has been pointed out previously (Quinton, '04, Adolph and Adolph, '25). This activity of the integument serves excellently in the normal medium, but adds a factor of susceptibility.

Existence in the freshwater medium is accompanied by the possession by all freshwater organisms of relatively dilute tissue fluids. Apparently it is the integumentary activity which is upset by increased concentrations of the medium. Its upset, in turn, produces rapid changes of a deleterious nature in the composition of the body fluids. Marine organisms, on the other hand, have body fluids which are in complete chemical equilib-

rium with their environments (Quinton, '04). Thus it turns out that while the essential physiological constitution of the tissues of the two groups of animals does not differ, the limiting factors for the proper functioning of their regulatory integuments differ vitally.

It seems probable that the transition from one type of integumental permeability to the other type occurs automatically during the long process of acclimatization which accompanies the transfer of an organism from one aquatic medium to the other; that both types are inherent in all integuments.

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ENDOCRINE GLANDS AND BILATERAL SYMMETRY: OBSERVATIONS UPON FORELIMB ERUPTION IN FROG LARVÆ UNDER TREATMENT WITH THYROID AND THYMUS EXTRACTS.

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INTRODUCTION.

A conspicuous feature of thyroid-accelerated metamorphosis in frog larvæ is the eruption of the forelimbs. Before these become visible externally they are located beneath the skin in the gill chamber. For some years the writer has been aware of the fact that the left forelimb is protruded before the right forelimb, sometimes by as much as several days. This condition was recorded in a previous paper (Jordan and Speidel, '23) and has probably also been noted by other workers in the field of amphibian metamorphosis. In half-grown bullfrog and green frog tadpoles no exceptions to this were seen; *i.e.*, no case appeared of precedent right forelimb eruption in thyroid-treated tadpoles. It was, therefore, of some interest to find in a jar of seven thymus-treated tadpoles one animal in which the right forelimb appeared two weeks before the left. Furthermore, in two other animals in this lot the amount of skin degeneration in the right forelimb region was definitely farther advanced than that on the left side, a condition indicating probable prior right forelimb eruption. Death ensued, however, before the appearance of either forelimb. Two of the other four animals in this jar put out the left forelimb first, and in one the amount of skin degeneration on the left side plainly foreshadowed the prior eruption of the limb of that side. The remaining animal put out both limbs over night. In this jar of seven thymus-treated animals, therefore, the ratio of "right-handed" to "left-handed" animals is 1 : 1.[1]

The question suggests itself as to whether the endocrine secre-

[1] The terms "right-handed" and "left-handed" are used to denote merely prior right forelimb eruption or prior left forelimb eruption, respectively.

tions represented by the thyroid and thymus extracts actually affect in a differential manner the bilateral symmetry of the developing animals. Further experiments were set under way in an attempt to analyze the factors controlling variation in forelimb eruption.

There is a difference of opinion as to the condition in normal frog larvæ. Barfurth ('87) in Europe finds in the case of *Rana fusca* that 80 per cent. put out the right forelimb first, the left almost always following in from two to eight hours. On the other hand, Gudernatsch ('14), using *Rana temporaria* and *Rana esculenta*, states that he has always found the reverse to be the case; *i.e.*, in about 80 per cent. the left forelimb erupts first.[2] Dickerson ('20) observes that in normal bullfrog and green frog metamorphosis the left forelimb is usually put out first. My own observations upon the normal tadpoles of *Rana sylvatica*, *Rana clamata*, *Rana catesbeiana*, and *Hyla crucifer* lead me to agree with Gudernatsch and Dickerson that in a definite majority of cases the left limb is the first to erupt.

Material, Experiments and Observations.

The material used includes about 800 tadpoles of *Hyla crucifer*, *Rana catesbeiana*, *Rana clamata*, and *Rana sylvatica*, and a few of *Rana cantabrigiensis* and *Rana pipiens*. The bullfrog and green frog tadpoles were collected at Charlottesville, Virginia, the others at Woods Hole, Massachusetts. Untreated animals were usually kept in aquaria containing pond water and weed. Administration of endocrine extract was accomplished by placing some of the extract in the water with the animals. Thyroid and thymus desiccated extracts were used.

The accompanying table indicates the relative frequency of left or right forelimb eruption, as it occurs under normal conditions, under thyroid treatment, and under thymus treatment.

[2] Gudernatsch makes this statement in a footnote referring to his experiment with thyroid-accelerated metamorphosis. It is probable, therefore, that he included his observations on thyroid-treated animals with those on normal animals in regard to forelimb eruption, not realizing that thyroid treatment affects forelimb eruption. Thyroid administration, as shown by this paper, markedly favors the prior eruption of the left forelimb. His percentage, therefore, is not correct, but is too high in favor of lefthandedness.

TABLE I.

In this table is given for each species of tadpole the number of individuals observed with prior left forelimb eruption as compared with the number of individuals observed with prior right forelimb eruption, under normal conditions, under thyroid treatment and under thymus treatment.[3]

	Normal-untreated.		Thyroid-treated.		Thymus-treated.	
	Prior Left.	Prior Right.	Prior Left.	Prior Right.	Prior Left.	Prior Right.
Hyla crucifer	43 (72%)	17 (28%)	25 (100%)	0	17 (50%)	17 (50%)
Rana sylvatica	42 (65%)	22 (35%)	19 (100%)	0		
Rana catesbeiana and *R. clamata*	3	0	85 (100%)	0	3 (50%)	3 (50%)
Rana cantabrigiensis			4 (100%)	0		
Rana pipiens			20 immature tadpoles, all died before either forelimb erupted			
Total, all species	88 (70%)	39 (30%)	133 (100%)	0	20 (50%)	20 (50%)

Under normal conditions a definite majority of tadpoles puts out the left forelimb first; in *Hyla* 72 per cent., in *Rana sylvatica* 65 per cent. The few observations upon untreated bullfrog and green frog tadpoles in the three-limb condition support Dickerson's observation that the left limb usually erupts first. Under thyroid treatment the left forelimb is invariably protruded first in all species studied, if those putting out forelimbs during the first forty hours of the treatment are excluded. Under thymus treatment apparently a 1 : 1 ratio is indicated. The experiments with *Hyla* afford the best comparative figures and may be taken as typical.

With thyroid treatment of half-grown tadpoles 100 per cent. put out the left forelimb first. A special experiment was tried in which 75 *Rana sylvatica* tadpoles were subjected to thyroid treatment, many of these at the time being on the verge of putting out the forelimbs. Among those animals putting out forelimbs within the first forty hours, seven righthanded ones were seen; thereafter all were lefthanded. It may be concluded that in animals protruding the forelimbs within this time, an original bias toward righthandedness may not be changed. In a similar experiment with 25 *Hyla* tadpoles that were also fairly close to

[3] For reasons explained in the text all animals putting out forelimbs within the first two days of thyroid or thymus treatment are discarded, and do not figure in the table.

the time of forelimb eruption, one righthanded animal occurred during the first 24 hours; after that all were lefthanded. For this reason, in Table I. all animals putting out forelimbs during the first two days are omitted from the reckoning, since the original normal bias may not have been sufficiently influenced by the endocrine extract.

How long it takes for the thymus extract to affect an original bias is not known. Since it is probably not so powerful as the thyroid it may be that more than the first two days' results should be discarded. If the first two days are discarded the ratio is 17 : 17 as given in the table. If the results of the first three to seven days are discarded the ratio shifts progressively to favor righthandedness. Only a much larger number of animals under observation would give a trustworthy ratio. The figures given, however, are in all probability enough to show that the normal ratio has been affected and shifted in the direction of righthandedness.

Interpretation and Discussion.

These observations leave no doubt that the bilateral symmetry of the developing frog tadpole, as indicated by the relative time of forelimb eruption, is influenced by thyroid extract; possibly also by thymus extract. The action of the thyroid extract will first be discussed. The blood carries the active thyroid principle to all parts of the body. It is inconceivable that the thyroid autacoid should have one effect on the tissues on the left side and another effect on exactly similar tissues on the right side. Therefore, the effect of the thyroid in changing the normal symmetrical development must be due to some original fundamental asymmetry of the body pattern.

The following findings are pertinent: The tadpole is conspicuously asymmetrical in respect to its respiratory apparatus. A spiracle, or outlet from the gill chamber, is present on the left side only (Fig. 1). This outlet drains both left and right gill chambers, these being connected by a canal across the mid-line. The forelimb is present in the gill chamber, its degree of development depending upon the general developmental state of the tadpole. Both in normal and thyroid-induced metamorphosis

the left limb is pushed through the spiracle, a variable amount of preliminary skin degeneration occurring. The right forelimb erupts through the skin only after the latter has undergone a certain amount of degeneration. This degeneration starts in the

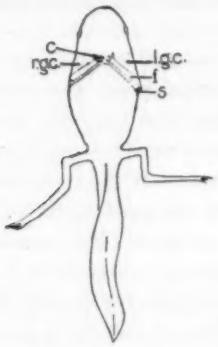

FIG. 1.

FIG. 2.

FIG. 1. Ventral view of a tadpole of the tree frog (*Hyla crucifer*) showing well developed forelimbs (*f*) still imprisoned beneath the skin in the gill chambers (*r.g.c.* and *l.g.c.*). The position of the sinistral spiracle (*s*) is shown, which drains both gill chambers; also the canal (*c*) across the mid-line which connects right and left gill chambers. The dotted line below the forelimbs indicates the position of the partition separating the gill chambers from the abdominal cavity.

FIG. 2. Dorsal view of a thyroid-treated tadpole (*Hyla crucifer*) in typical three-limb stage after the eruption of the left forelimb. Collection and retention of air (*a*) in the right gill chamber causes bulging out of the skin and interferes with forelimb eruption on that side. Excluding the first two days, prior left forelimb eruption occurs invariably.

vicinity of the elbow, and the elbow is usually protruded first. Movements of the forelimb finally enable it to break completely through. In young thyroid-treated tadpoles, however, the forelimbs are little developed so that the elbow is not prominent at the surface. In these cases the hand or whole arm appears as a tiny white stump after the skin degeneration has proceeded far enough.[4]

The administration of thyroid extract to a half-grown tadpole obviously upsets the respiratory mechanism. More air is taken

[4] While this paper was in press, Helff ('24), at the Washington meeting of the American Society of Zoölogists, reported a series of experiments which show clearly that the opercular skin autolysis preceding forelimb eruption is brought about by substances given off by the adjacent atrophying gills.

in. But apparently the animal is not yet very well fitted for utilizing it properly. Small bubbles of air collect in the gill chambers and are not expelled, or expelled with difficulty. This is especially true of the right gill chamber which has no outlet except through the left spiracle (and, of course, the mouth). This proves to be the deciding factor. The left gill chamber is usually drained well enough except in very immature tadpoles, so that skin degeneration and forelimb eruption on that side are not interfered with. On the right side, however, the retention of air in the chamber causes bulging out of the skin (Fig. 2), and interferes to a greater or less degree with the normal eruption on that side, thus bringing about the typical prior left forelimb eruption. A tadpole in this stage does not present a normal posture when at rest, but floats with the right side somewhat elevated owing to the air in the right gill chamber. The difference between a thyroid-treated animal in this condition and either normal or thymus-treated animals may be seen by comparing Fig. 2 with Figs. 3 and 4. Occasionally, in very young

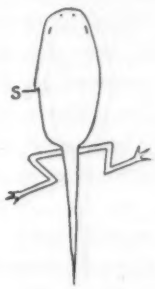

FIG. 3.

FIG. 4.

FIG. 3. Dorsal view of a normal tadpole (*Hyla crucifer*) before the eruption of either forelimb. The location of the spiracle (*s*) on the left side only, leads to a majority (70 per cent.) of prior left forelimb eruptions.

FIG. 4. Dorsal view of a thymus-treated tadpole (*Hyla crucifer*) showing prior right forelimb eruption. Either right or left forelimb may erupt first. In the region of the spiracle (*s*) may be seen an angular projection (*p*) caused by the pressure of the imprisoned left forelimb.

and immature tadpoles no forelimbs erupt with thyroid treatment, the animals dying in the two-limb stage. In these, bubbles of air may be seen in both gill chambers. The pulmonary

development of the animal is not adequate to the demands imposed upon it by thyroid treatment and death results. Coupled with this respiratory disturbance is also the condition of anemia already pointed out elsewhere (Jordan and Speidel, '23). The older the tadpole the more probable it is that both forelimbs will erupt. The pulmonary apparatus is presumably better developed, and the forelimbs are large enough so that limb movements aid both in expelling the air from the gill chamber and in breaking through the skin. In thyroid-treated animals that are near the time for metamorphosis both forelimbs are put out with little trouble, the sinistral location of the spiracle becoming of less importance.

In a recent paper by Swingle ('23) one figure is given to show the effect of iodo-tryosine administration in accelerating metamorphosis in pituitaryless *Rana sylvatica* tadpoles. After sixteen days of treatment the specimen illustrated has one forelimb, that one being a right forelimb. Swingle does not state when this particular right limb appeared but does say that two right forelimbs broke through as early as the eighth day, the average, however, being about twenty days. This is an interesting observation in comparison with my results after thyroid treatment; *i.e.*, 100 per cent. prior left forelimb eruption after the first two days. It seems to mean, either that iodo-tryosine does not affect the respiratory apparatus in the same way as does thyroid extract, or that the pituitary gland plays a rôle also in influencing symmetrical development.

It now remains to discuss the normal condition and the thymus-treated condition. Three factors are considered of chief importance in determining forelimb eruption: (1) sinistral location of the spiracle; (2) relative degree of skin degeneration over the forelimb region on the two sides of the body; (3) relative size and strength of the forelimb. In partly grown thyroid-treated animals the first factor is by far the most important, as has been shown. In mature untreated tadpoles, however, this factor does not remain the all-important one. The forelimbs are now so large that the left one cannot be pushed through the spiracle without a fair amount of previous skin degeneration. Simultaneous skin degeneration occurs on both sides. The size, strength and activity of the imprisoned forelimb now becomes of

much importance. In about 30 per cent. of cases the right forelimb succeeds in overcoming the handicap of having no spiracle to come through, and breaks through by main strength before the left. In the other 70 per cent. the left limb aided by the sinistral spiracle comes through first. A majority in favor of lefthandedness is about what should be expected.

Mention should be made again of Barfurth's results. In normal *Rana fusca* tadpoles he finds that 80 per cent. put out the right forelimb first. Presumably these are like all other frog tadpoles in having sinistral spiracles although Barfurth does not refer to this feature. He believes the prevailing righthandedness of this species is accounted for by two factors: (1) earlier and greater degeneration of the skin in the right forelimb region, and (2) greater size and strength of the right limb. As these results are directly opposed to the observations of Gudernatsch, Dickerson and myself on five species of frog tadpoles, it can only be supposed that there is a species difference, and that the factors mentioned by Barfurth are strong enough to bring about a majority of righthanders in this particular species. It would be of interest to see whether in this species also the uniform lefthanded condition could be produced by thyroid treatment.

The explanation of the results after thymus treatment is somewhat more difficult and uncertain. It is probable that a shift toward righthandedness is here indicated. The ratio may be 1 : 1, although on account of the small number of animals observed in the three-limb stage, this is by no means a certainty. At any rate, the lessening of the lefthanded majority means that the asymmetrical position of the spiracle becomes of much less importance as a factor in determining the first forelimb to appear.

Desiccated thymus extract is a food rich in nutritive value, and therefore favorable to growth. Gudernatsch ('14) noted its growth-promoting effect upon tadpoles and ascribed it to the endocrine secretion of the thymus. Uhlenhuth ('17) though he combats Gudernatsch's idea as to the growth effect being due to an endocrine secretion contained in the thymus extract, states that it is a very rich and nutritious food and therefore quite favorable to growth. The writer has also observed that it is particularly favorable to limb growth. In one batch of partly-grown thymus-treated green frog tadpoles the small hind limbs

became quite red and vascular and grew rapidly, almost reminding one of the effect of thyroid extract. The writer became suspicious of the thymus extract used and had it analyzed for the presence of iodine.[5] The analysis gave negative results. The later history of the tadpoles showed that only growth in size of the larval structures was being stimulated, and not differentiation. There was no acceleration in skin degeneration of the forelimb region, except that caused secondarily by pressure of the growing forelimb. There was likewise no reduction in the tail, but on the contrary growth. With thymus treatment limb growth appears to proceed relatively faster than the general process of body differentiation. As a result, the forelimbs enclosed beneath the skin reach a comparative size and strength, such that they become the important factor in determining the time of eruption. With increasing limb size the spiracle becomes less important since the forelimb cannot be pushed through it without complementary skin degeneration. Since size of forelimb and amount of skin degeneration on the two sides are about equal in the species under observation a more equal ratio of righthandedness to lefthandedness results.

The writer does not wish here to enter the controversy as to whether or not the thymus extract has a specific endocrine content. Its effect upon symmetry in forelimb eruption in tadpoles seems to be best explained on the grounds given above; *i.e.*, its unquestioned value as a highly nutritious and therefore growth-promoting food. It is not necessary to assume a specific endocrine effect. This much, however, may be added. Thymus gland is largely lymphoid tissue. In the light of Carrel's work showing the growth-promoting effect of leucocytic secretions or "trephones" ('24), and the confirmatory observations of Jordan and Speidel ('23) on lymphocytes in rapidly growing regions in tadpole metamorphosis, it would seem probable that growth-promoting substances (trephones) of lymphocyte origin would be present in thymus extract.

In conclusion, it may be pointed out that these observations and their interpretation, though of little importance in themselves, suggest the possibility of the following principle operating

[5] The analysis was made by Mr. T. F. Otto, of the University of Virginia Medical School.

in any vertebrate animal in process of development: A change in the normal balance of thyroid secretion may lead to a change in the symmetrical development. Stockard ('23) has emphasized the general importance of thyroid secretion in the development of man and mammals and its part in determining the production of definite types. These results on forelimb eruption in tadpoles indicate that thyroid secretion may be of some importance also in influencing symmetrical development. A vertebrate animal, though designated as bilaterally symmetrical, is, of course, asymmetrical in many respects, *e.g.*, the visceral pattern, much of the vascular system, etc. Given an original asymmetrical condition it is possible that the thyroid may exert its effect upon the two sides of the body in a differential way.

Summary.

The bilaterally symmetrical development of the frog larva is affected in a definite way by experimental hyperthyroidism. Normally in tadpole metamorphosis the left forelimb erupts first in about 70 per cent. of cases. With thyroid-accelerated metamorphosis of half-grown tadpoles practically 100 per cent. will put out the left forelimb first. Of 133 thyroid-treated animals of this kind every one protruded the left forelimb first. In the case of full-grown tadpoles already near the time of forelimb eruption, thyroid treatment may be followed during the first two days by some prior right forelimb eruptions; thereafter prior left forelimb eruption obtains. In other words, an original bias of an animal toward prior right forelimb eruption may not be changed by thyroid administration within two days.

This effect of the thyroid on symmetrical development is explicable in terms of the original asymmetrical pattern of the respiratory apparatus (*i. e.*, sinistrally located spiracle) coupled with the close anatomical relation of the forelimb to this apparatus.

Thymus treatment brings about a larger percentage of prior right forelimb eruptions, thus reducing somewhat the normal majority in favor of prior left forelimb eruption.

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CELL SIZE AND METABOLIC ACTIVITY IN AMPHIBIA.

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I. INTRODUCTION.

The fact that the mass of a body increases as the cube of the linear dimension, while the surface increases as the square, has long been recognized as of importance in biology. Leuckart (1852) uses it to explain the necessity for increased surface as an organism becomes larger, a necessity which is met in animals by inpushings and the develompent of a distributing system; in plants by outgrowths. Herbert Spencer (1873) says: "Why has the individual a growth limit? . . . In similarly shaped bodies, the masses vary as the cube of the dimensions, whereas the strengths vary as the square of the dimensions." He applies this idea to an individual whose height doubles in a given growth period; the mass has been multiplied by eight, but the strengths of muscles and bones, being proportional to their cross section, are multiplied by only four. The absorbing surface is also multiplied by four, while the mass to be nourished by the material absorbed is multiplied by eight. It is only a step farther to apply the same idea to cell size. The writer was unable to learn who first did this.

Since the absorbing surface of a cell increases only twice as fast as the radius, while the mass to be nourished by absorbed material increases four times as fast, it follows that there is a definite size limit to cell growth; and further it is evident that the absolute size attainable by any given cell is inversely proportional to its rate of consuming the material absorbed. In other words, a sluggish cell may absorb the relatively small amount of material needed for its activity through a smaller surface than a more active cell, with its greater requirements of material; or the sluggish cell could grow to a larger size than the active one and still get sufficient material through its surface. If this is true, then one may reasonably expect a sluggish animal to have larger cells, while a more active animal would require smaller cells.

The research described in the present paper was started with the idea of obtaining some experimental data which might indicate whether the assumption stated above is true, and therefore whether the size of cells might be of fundamental importance in the activities of an animal. The Amphibia were chosen for experimental material because they have quite large cells which can be measured with less error than smaller cells, and because the Amphibia are known to vary quite widely in both cell size and activity.

Gulliver (1875) published measurements on the blood corpuscles of 650 species of Vertebrata; including 3 Cyclostomata, 11 Elasmobranchii, 75 Pisces, 17 Amphibia, 38 Reptilia, 265 Aves, and 241 Mammalia. Perusal of his figures with a consideration of the relative degree of activity of the various animals indicates a general agreement with that to be expected if cell size does vary inversely with activity, but there are numerous exceptions. Most of these exceptions can be explained on the basis of the size of the animal, for Gulliver points out that, within a limited group, the size of the red blood corpuscles increases with increasing weight of the members of the different species considered. That activity may be of importance in connection with cell size is indicated by the fact that, among the Mammalia, the smallest corpuscles are found in the deer family, the largest in the elephant, porpoise, anteater, and sloth and near the average size among the Carnivora. Among the Cheiroptera the

fruit-eating bats have distinctly larger corpuscles than the insect eaters. Among the birds the largest corpuscles are found among the Cursores, and the smallest among the insect-eating passeriform birds. Among the reptiles the Chelonia have distinctly larger corpuscles than the Sauria. The corpuscles are much larger in the Caudata than in the Salientia, and larger in the Elasmobranchii than in the Pisces.

Other workers who have given measurements of red blood cells are Weckler (1863), Malassez (1872), Formad (1888), Wormley (1888), and Forrest (1913). Forrest and Malassez also made counts. These run in inverse ratio to the size, although there are exceptions. A comparison of the measurements of amphibian corpuscles by different workers will be found later. Reichert and Brown (1909) review the work which has been done on red blood cell size and state that attempts to correlate the size of these cells with the rapidity of the animals' movements are founded on insufficient or erroneous data.

Hartmann (1919*a*) shows that the chloroplasts in developing *Elodea* leaves are smaller and more numerous in plants grown at higher temperatures, as contrasted with the larger and less numerous chloroplasts in plants grown at lower temperatures. Since the metabolism of the leaves is certainly speeded up with increased temperature, this observation falls well in line with the idea that a high rate of activity is associated with small size.

Chambers (1908) shows that there is considerable variation in the size of the eggs of *Rana esculenta* and *R. temporaria*, that the larger eggs develop a little more rapidly than the smaller ones, and that there is a much higher percentage of mortality among small than among large eggs, especially when grown at higher temperatures. He shows that the size of the cells in the frog varies with the size of the egg from which the frog developed; and that eggs allowed to develop at higher temperatures invariably yield smaller frogs with smaller cells than those developed at lower temperatures (size taken at time of metamorphosis or earlier). Tadpoles in crowded cultures are smaller than those with more room, but this does not affect the size of the cells. Morgan (1904) worked on dwarf frog eggs which had only about half the volume of the normal eggs, and showed that the cells in the developing dwarf embryos tend to remain smaller

than normal. Berezowski (1910) worked on the size of the intestinal cells of the white mouse during development, and shows that these cells become larger as the animal grows.

Krogh (1916) gives comparative tables on basal metabolism as it has been worked out by various workers on different animals. These figures indicate that there is a general agreement between activity as measured by basal metabolism, and cell size as measured on the red blood cells by Gulliver (1875), in inverse ratio. Figures given by different workers on metabolism vary quite widely, and this is true to an extreme degree of Amphibia. For instance Regnault and Reiset (quoted in Morat and Doyon, 1900) found that 0.063 mg. of CO_2 was eliminated per gram of frog per hour, while Krogh (1916) gives a figure which corresponds to 0.3686 mg. of CO_2 per gram per hour.

II. Material and Methods.

As many different species of Amphibia were used as it was possible to obtain. Activity was measured in terms of carbon dioxide output. This was measured by fixing the gas as a precipitate of barium carbonate in a barium hydroxide solution. In detail the method consisted in sucking air by means of a filter pump through one 8-inch tube of concentrated potassium hydroxide, two 8-inch tubes of soda lime and a gas washing bottle containing strong barium hydroxide. This series was to remove the carbon dioxide from the atmospheric air. The stream of air then passed into a respiration chamber containing the animal. Even when air breathing animals were used some water was always placed in the bottom of this chamber to keep the skin of the animal moist. From the respiration chamber the air current passed through two or three gas-washing bottles containing a carefully measured amount of standardized barium hydroxide. Special care was taken to see that the air was broken up into fine bubbles as it passed through these bottles. To accomplish this the end of the inlet tube was drawn out into two fine points. The bulb type of bubbler was found unstaisfactory because it was too easily broken in the numerous manipulations incident to making a long series of determinations; and because a finer stream of bubbles could be obtained by the method described. Suction tubing was used in making connections, and special precautions were taken to avoid leaks.

Standard solutions were made by preparing a stock solution of N/10 oxalic acid by weight, N/10 barium hydroxide standardized against this, and N/10 hydrochloric acid standardized against the barium hydroxide. Phenolphthalein was used as an indicator in preparing the standards and in the actual determinations. A preliminary aëration was run with the animal in the respiration jar, but without the collecting bottles, for one hour. The collecting bottles were then placed in the series, and aëration carried on for a measured length of time, 8–24 hours. At the end of this aëration the collecting jars were removed and the excess of hydroxide titrated immediately by means of N/10 hydrochloric acid. The amount of hydroxide used by the carbon dioxide was thus obtained by difference, and the amount of carbon dioxide collected computed as CO_2 per gram body weight per hour. All determinations were made on starving animals and at room temperature, which varied between 20 and 23° C.

Truog (1915) describes a method of determining carbon dioxide by passing air through a tower containing barium hydroxide and glass beads. This method would be more accurate than the one used here, but the method was unknown to the writer at the time when the experiments described in this paper were undertaken. It is felt that the method used here yields results of comparative value, which is all that is needed. Truog shows that the barium carbonate present with the hydroxide does not hinder accurate titration, and that the barium hydroxide method of determining carbon dioxide is very accurate.

Red blood corpuscles were used for measuring cell size. Most of the animals used were those on which carbon dioxide determinations had already been made. The animal was killed either by pithing or with chloroform, and blood taken either from the heart by means of a syringe or from the tail vein. Thin smears were made on slides, dried in the air and stained with Wright's stain. In many cases blood counts on both red and white corpuscles were made, and tissues fixed in Bouin's fluid for section later in order that other cells might be measured. The blood counts and tissue cell measurements are not included in the present paper. The latter agree reasonably well with the results given for red blood cells, while blood counts are found to vary widely with the physiological condition of the animal. Of course in general animals with larger cells have smaller numbers.

Red blood cells were measured by means of a scale which was so constructed that each division on the scale corresponds to one micron in the oil immersion field (B. & L. 1.9 mm. obj.) with 10x ocular and 160 mm. tube length, when the scale is placed on the table beside the microscope and viewed through a camera lucida with mirror set at an angle of 45° and the arm length set at 103 mm. With the aid of this scale the dimensions of red blood cells were measured, 50 cells being measured from each slide, and from one to five slides being used for each animal studied. From the average length and width thus obtained the surface of the average corpuscle for each animal was computed, assuming no thickness, from the formula $\pi LW/2$ where L is the maximum and W the minimum diameter. This formula follows from the formula for a regular ellipse, πab, where a and b are the long and short radii. The surface is used as the significant figure rather than the length and width because all corpuscles are not the same shape, length and width have different ratios, and therefore the dimensions do not give a direct index of size.

III. Results for the Different Species of Amphibia.

Table I. gives the results for the various species used. Where a space is left blank, no data were obtained on the particular point concerned. For instance it will be noticed that sex is not given in a number of cases, and in the same cases usually no blood cell measurements are given. In these cases the animal died and it was not considered safe to make blood cell measurements on such animals in which post mortem changes had had time to occur. Therefore they were not autopsied at all, and thus no data were obtained on sex. In several species it was impossible to obtain samples for carbon dioxide determinations, although one or several had already been used for blood smears.

A short description of the material and results for each species is given below. The species are taken in the same order as in the table; that of red blood cell size.

Amphiuma means (Gordon).—This species has the largest corpuscles known for any amphibian. Three adult specimens and one young were obtained from New Orleans. Measurements of carbon dioxide output were made at intervals over a period of two weeks. The animals showed evidence by their

TABLE I.

CELL SIZE AND CARBON DIOXIDE PRODUCTION FOR THE DIFFERENT INDIVIDUALS USED IN EACH SPECIES STUDIED.

Specimen, Sex and No.	Weight, Grams.	CO. Mg. per Gm. Wt. per Hour.	Number of Trials.	Red Blood Corpuscles.			
				Number Measured.	Micra Length.	Micra Width.	Sq. Mic. Area.
Amphiuma means.							
F 1.....	large			50	62.8	35.42	3.494
F 2.....	41.4	0.0920	3	150	60.9	34.8	3.329
3.....	1,243	0.02136	3	50	62.18	36.8	3.594
4.....	1,270	0.02146	3	50	62.06	37.42	3.648
5.....	1,500	0.02125	2	50	61.0	39.04	3.741
Necturus maculosus.							
F 2.....	154	0.0420	5	250	53.78	30.8	2,602
M 3.....	194	0.0413	5	250	57.45	30.62	2,763
F 4.....	126	0.05285	16	250	54.568	27.496	2,357
M 5.....	130	0.0548	4	250	50.152	26.012	2,049
M 6.....	115	0.0532	10	150	52.386	26.46	2,177
M 7.....	126	0.05576	13	25	55.52	29.24	2,550
F 8.....	57.6	0.0922	12	250	51.144	30.984	2,489
M 9.....	55.7	0.1069	13				
M 10.....	86	0.0883	1	50	54.44	30.54	2,612
11.....	47	0.1399	2				
F 12.....	75	0.0915	1				
F 13.....	93.5	0.0842	2				
M 14.....	92	0.0753	1	50	54.04	25.7	2,187
15.....	74	0.1051	1				
F 16.....	44.3	0.1208	3	50	50.38	31.66	2,505
F 17.....	64	0.1192	2	50	53.72	25.28	2,133
18.....	194	0.0599	1				
Cryptobranchus alleganiensis.							
1.....				50	41.12	23.16	1,496
Diemyctylus viridescens.							
1.....				50	29.64	17.76	827
Rana catesbiana							
F 1.....	455	0.0629	4	250	25.2	13.032	516
F 2.....	604	0.0527	3	250	27.324	13.444	578
F 3.....	714	0.05257	4	50	25.58	12.86	517
F 4.....	510	0.0929	5	50	25.62	16.18	651
M 5.....	361	0.0671	3	250	26.836	13.176	555
6.....	503	9.0584	3				
M 7.....	388	0.0704	3	50	25.38	14.14	564
8.....	492	0.0627	3				
M 12.....	507	0.0876	3	50	24.48	13.84	532
F 13.....	411	0.0699	3	100	26.67	16.82	702
14.....	361	0.0787	3				
F 15.....	628	0.0843	3	50	23.86	14.16	531

TABLE I.—*Continued.*

Specimen, Sex and No.	Weight, Grams.	CO_2 Mg. per Gm. Wt. per Hour.	Number of Trials.	Red Blood Corpuscles.			
				Number Measured.	Micra Length.	Micra Width.	Sq. Mic. Area.
Rana clamitans.							
1.....				530	22.072	12.498	433
F 2.....	19	0.1794	4	250	23.236	12.22	446
M 3.....	7.1	0.1359	4	100	23.64	11.03	409
Rana pipiens.							
F 1.....	43.4	0.1386	9	100	19.53	12.56	385
M 2.....	25.4	0.1422	17	150	20.37	11.32	362
F 3.....	45.8	0.1768	7	250	21.078	14.192	470
M 4.....	27.0	0.2026	5	250	22.96	13.644	492
M 5.....	30.5	0.1762	13	50	19.86	13.78	421
M 6.....	33.6	0.1394	10	50	21.24	12.96	432
F 7.....	59.0	0.1795	3				
F 8.....	43.5	0.1931	6	50	18.46	13.42	389
F 12.....	54.7	0.1145	2				
F 13.....	52.6	0.1314	1				
M 14.....	32.7	0.1103	1				
F 15.....	40.8	0.1765	5				
M 16.....	28.9	0.2002	7				
M 17.....	14.4	0.2037	1				
Rana palustris.							
1.....				100	20.47	13.94	448
F 2.....	5.7	0.2742	5	200	20.11	12.705	401
M 3.....	3.6	0.2918	8	250	19.12	12.16	365
F 4.....	3.7	0.2531	9	250	20.384	12.152	389
F 5.....	3.9	0.1710	2	100	21.58	12.44	422
6–17....	36	0.1603	2				
18.....				50	20.7	13.96	454
M 19.....				150	19.13	12.47	375
Hyla pickeringii.							
1.....				50	19.44	11.2	342
Acris gryllus.							
1.....				50	17.82	11.18	313
2.....				50	17.7	10.6	295

TABLE I.—*Continued.*

Specimen, Sex and No.	Weight, Grams.	CO_2 Mg. per Gm. Wt. per Hour.	Number of Trials.	Red Blood Corpuscles.			
				Number Measured.	Micra Length.	Micra Width.	Sq. Mic. Area.
Chorophilus nigritus.							
1.....				50	17.96	10.04	283
Bufo americanus.							
F 2.....	11	0.1831	5	250	16.044	9.684	244
3.....	1.9	0.2069	1				

feces of having eaten recently. The baby specimen was killed and blood smears made immediately at the termination of the carbon dioxide output measurements; the adults were bled from the tail vein after six weeks.

Necturus maculosus (Rafinesque).—All specimens except the first two used were collected from Lake Mendota at Madison. They were kept in cold running water without food until used. From the time of the first determination on an individual until it was killed or died it was kept at room temperature. The first two specimens were received from dealers, and their source is unknown. In this species starvation was carried on over a long period of time, and its effect on metabolism studied. Individuals of widely varying weights were used and the effect of weight on metabolism noted. The results of these experiments will be described later.

Cryptobranchus alleganiensis (Daudin).—The single specimen used was a laboratory specimen of unknown source from which blood smears were made. No individuals were available when needed for metabolism tests on account of the cold weather.

Diemyctylus viridescens (Rafinesque).—One specimen was used for blood smears. It was collected in New York state during the summer, and blood smears were made in the fall. Probably the animal had not eaten in the meantime.

Rana catesbiana (Shaw).—Twelve specimens were obtained from New Orleans. Specimen No. 1 had had the lower jaw broken at some previous time, and it had healed in such a manner

that eating was impossible and the animal was in an extreme state of starvation. The other specimens showed signs of having recently eaten, and were well fed. They were kept at a temperature of about 12° C. when not being used for carbon dioxide output tests. Attention is called to specimen No. 4, which shows an unusually high carbon dioxide output. It is not included in the general average for the species in Table VIII. The detailed record for this animal follows: 2/10—0.0653, 2/29—0.0639, 3/20—0.1208, 4/18—0.1037, 4/20—0.1110 (dates of determinations and carbon dioxide output figures). No explanation is found for this strange behavior; starting with approximately a normal carbon dioxide output value, jumping to twice the normal, and remaining there. That the phenomenon is not due to an acute infection is indicated by the fact that the animal appeared normal on autopsy a month after the final test.

Rana clamitans (Latreille).—Two specimens were collected near Madison and kept at room temperature until used three months later. They had no food during this time. Blood smears were made from No. 1 immediately after it was collected. The low value for carbon dioxide production with the small animal is probably due to the extreme emaciation of this specimen. Neither cell size nor activity results differ essentially from those of the following species.

Rana pipiens (Shreber).—The specimens used were from several shipments from supply houses. Some of these animals were starved at room temperature for a long time, and the effects of lack of food on metabolism were noted. No appreciable decrease in metabolism resulted until the animal had reached an extreme state of starvation. No. 2 lost 47 per cent. of his body weight before any marked drop in carbon dioxide output per gram of body weight was obtained. Several specimens died of disease. The results of the study of metabolism during the course of the disease will be described later.

Rana palustris (Le Conte).—The specimens used were collected near Madison in the late fall. They were kept at room temperature and used for carbon dioxide output tests over a period of two months. At the end of this time they were all very weak from starvation. Nos. 1, 2, and 3 were killed while still in good condition. No. 5 and Nos. 6–17 were tested when near death

from starvation. No. 18 was starved, while No. 19 was used for blood smears within a few days after being collected.

Hyla pickeringii (Holbrook).—One specimen was collected and blood smears made before the carbon dioxide tests had been started. None were available while tests on metabolism were being made.

Acris gryllus (Le Conte).—Two specimens were collected from the field and blood smears made. Time was lacking to make carbon dioxide output tests.

Chorophilus nigritus (Le Conte).—See note on *Hyla pickeringii*.

Bufo americanus (Le Conte).—Two specimens were collected and starved at room temperature three months before using. No. 3 died of starvation after only one test for carbon dioxide had been made, and blood could not be obtained for smears.

IV. Controls.

A. Cell Size.

1. Blood corpuscles may shrink as smears dry. However the same methods were used on all specimens, so that the results should be comparable. That there is a certain amount of differential shrinking due to differences in thickness of smear is suggested by comparing measurements from different slides made from the same individual. For instance the five slides of blood from Rana clamitans No. 2 gave the following series of averages of 50 corpuscles from each slide: 23.98 x 13.02, 22.92 x 11.66, 23.32 x 12.08, 23.1 x 12.32 and 22.86 x 12.02. Some of this variation may have been caused by differences in the corpuscles which happened to be measured, but most of it was probably due to the nature of the smear. With the slide which averaged 23.98 x 13.02 the first 25 corpuscles measured 23.84 x 13.12, while the last 25 measured 24.12 x 12.92. The method used is that of Gulliver (1875) except for the fact that he does not appear to have stained his smears. Georgopolus (1906) states that dry preparations are unreliable because the size of the cells is likely to vary with the thickness of the film, and states his preference for the wet method. This consists in placing a small drop of fresh blood on a clean slide and quickly placing on a cover. The corpuscles are then measured immediately. This method has been found useless for Amphibia because the corpuscles are distorted by the

treatment. For the smaller mammalian corpuscles it is an excellent method. The one attempt which was made to use the wet method gave sizes which checked fairly well with measurements from dried smears. It was found to be difficult to use an oil immersion objective on a wet preparation, and it was difficult to find corpuscles which were not distorted.

For convenience of reference for the reader, and to show how the present measurements check with those given by other workers, Table II. has been prepared to show the sizes of am-

TABLE II.

MEASUREMENTS ON AMPHIBIAN RED BLOOD CORPUSCLES AS MADE BY DIFFERENT WORKERS.

Species.	Gulliver.	Weckler.	Wormley.	Forrest.	Morat and Doyon.
Amphiuma tridactylum (means)	69.9 x41.3		70.9x40.9		
Proteus anguineus	63.5 x34.94	58.2x33.7			58.0x35.0
Siren lacertina	60.47x33.42				
Cryptobranchus japonicus	56.45x31.75				
Cryptobranchus alleganiensis	45.11x25.4				
Siredon humboldtii	44.8 x25.4				
Lissotriton punctatus	31.75x19.84				
Salamandra				37.8x23.8	
Triton bibronii	29.95x19.84	29.3x19.5		29.3x19.5	29.3x19.5
Triton cristatus	29.95x19.84				
Rana		22.3x15.7	23.3x14.1	22.3x15.7	22.3x15.7
Rana esculenta	25.4 x17.58				
Rana temporaria	22.92x13.95				
Bufo		30.2x18.2		21.8x15.9	
Bufo vulgaris	24.35x12.7				
Bufo clamita	19.05x13.4				

phibian corpuscles according to the measurements of other workers. The writer's measurements are not included here because they are mostly on different species, the table would therefore be much longer, and the measurements are found elsewhere in this paper.

2. It is possible that some of the animals studied by the writer show abnormally small corpuscles on account of extreme starvation. *Necturus* No. 6 and *Rana pipiens* No. 2 are cases with extreme starvation and small cells. However, *Necturus* Nos. 7 and 8 and *Rana pipiens* No. 5 also underwent extreme starvation,

and show relatively large cells. *Rana palustris* No. 19 was collected from the field and blood smears made immediately, yet this specimen shows the smallest corpuscles measured for this species. On the other hand *Rana palustris* No. 18 shows the largest corpuscles of the species, and was used when in an extreme state of starvation. There seems to be no correlation between cell size and degree of starvation, as judged by the size of the red blood cells.

B. Carbon Dioxide Determination.

1. The apparatus used has two inherent defects. These are not considered to be of sufficient importance to affect the results for the relatively large amounts of carbon dioxide measured. They are, first that rubber tubing was used for all connections, and second that soft glass bottles were used for collecting jars. Rubber tubing has been shown to have a selective absorption for carbon dioxide, but this should not be important considering the short lengths of tubing used, the rapidity of the air stream, and the relatively large amounts of carbon dioxide collected.

2. To learn whether the traps to remove carbon dioxide from the air before it entered the respiration chamber were taking out all the gas, a gas washing bottle containing a carefully measured amount of standard barium hydroxide was placed between the traps and the respiration chambers. 22 hours of rapid aëration yielded 5.06 mg. of CO_2, or 0.23 mg. per hour.

3. To learn whether some of the expired CO_2 was getting by the collecting jars, a barium hydroxide bottle as in the previous case was placed between the collecting jars and the pump. A rapid stream of air passing through two respiration jars and two sets of collecting jars (in parallel; one for *Necturus*, one for *Rana pipiens*), was sucked through this jar for 26 hours. 28.6 mg. of CO_2 were collected, or 1.1 mg. per hour. Subtracting from this figure the amount introduced into the jars with the inhaled air, it appears that 0.87 mg. per hour of CO_2 was being lost from the system. The animals in the jars weighed 93 grams, so that the loss is 0.00936 mg. per hour per gram weight of the animals. This is about an 8 per cent. loss for *Necturus* and about 6 per cent. for the frog. For the larger animals an additional collecting jar was used. This of course tended to keep down the loss.

4. Leaks were practically eliminated. It was possible to develop a strong negative pressure in the jars, close all valves, and allow the apparatus to stand for several hours with no appreciable diminution of pressure.

5. The negative pressure in the respiration jars averaged about 2 cm. of mercury. This factor was practically constant for the entire course of the experiments.

6. Measurements of the rate of flow with the air current moving at as near the average rate as was possible to judge yielded two minutes for each liter of air. This stream was divided between a jar of four liters capacity (used for *Necturus*, *Rana catesbiana* and *Amphiuma*) and one of one liter capacity (used for the smaller animals). Tests on each jar separately showed that the air in the large jar was being changed every eleven minutes, while that in the small jar was changed every five minutes. This should be sufficient speed in each case to keep the atmosphere around the animal relatively free of carbon dioxide. Attempts to cut down the rate through the small jar to more nearly equal that of the larger jar were unsuccessful because it was found that, with too slow a rate, the holes in the bubblers in the gas collecting jars became clogged with precipitate of barium carbonate and the aëration stopped.

7. Determinations of the dissolved carbon dioxide in the water surrounding the animal at the end of the preliminary aëration and again at the end of the final aëration yielded approximately the same figure in each case. For instance, while a carbon dioxide test was being made on a bull frog, no titratable CO_2 was found in the water used in the jar, 5.5 mg. CO_2 per 100 cc. were present in the water at the end of the preliminary aëration, and the same figure at the end of the final aëration.

8. Several blanks were run with the regular amount of water in the respiration jars, but without animals, and one with a completely empty jar. The results of these trials are shown in Table III.

For all the above tests 250 cc. of N/10 barium hydroxide were measured into two gas-washing bottles, and the bottles filled to capacity (300 cc.) with distilled water. This same procedure was followed in filling the jars for the regular carbon dioxide output tests. For numbers 7 and 8 solutions were used which

were not quite standardized. Therefore No. 8 is of value only in comparison with No. 7. Subtracting the two it is found that the 14 hours of aëration resulted in the accumulation of 11 mg. of CO_2, or 0.785 mg. per hour. Comparing this with No. 6 it is seen that 1.275 mg. per hour resulted from the presence of water in the respiration jar in the aëration numbered 6. The source of this CO_2 is the dissolved bicarbonate of the water, which gradually liberates carbon dioxide when CO_2 free air is bubbled through it. This is not a factor when an animal is in the jar, as is shown by the determination recorded above of the amount of dissolved CO_2 in the water surrounding a respiring animal; sufficient to prevent liberation of the gas from the bicarbonate. Trials Nos. 1, 2, 3 and 5 indicate that this combined CO_2 comes out quite slowly. No. 4 represents the degree of accuracy which can be expected from the titration. This figure represents 0.8 cc. of N/10 barium hydroxide in 250 cc.; and thus is an error of 0.32 per cent.

TABLE III.

BLANK TESTS ON CARBON DIOXIDE.

Trial No.	Hrs. of Aëration.	Mg. CO_2 Collected.	Mg. CO_2 per Hour.	Remarks.
1	5	5.02	1.012	Small jar. One liter capacity.
2	9	5.72	0.635	Small jar.
3	14	8.36	0.597	Small jar.
4	0	1.76	—	Treated as for aëration, but titrated immediately.
5	27	11.98	0.444	Small jar.
6	24	49.5	2.06	Large jar. Four liters cap.
7	14 (24)	18.48	0.486	No water. Large jar, 14 hrs. aëration plus 24 hrs. standing.
8	(38)	7.48	0.197	Parallel with No. 7. No aëration; standing.

It appears, then, that the carbon dioxide collected in the blank aërations is from three sources: (1) that due to the liberation of the bound CO_2 of the water, (2) that caused by residual CO_2 in the atmosphere of the respiration chamber at the end of the preliminary aëration, and (3) that resulting from CO_2 getting through the air washing system before the air enters the respiration chamber. Of these only the last is of importance in producing error, the others being eliminated by the presence of a

respiring animal in the jar. This factor has already been shown to be overcompensated by the loss resulting from incomplete absorption of the carbon dioxide by the barium hydroxide. Inasmuch as the first two factors are important, they would tend to neutralize this loss. It is therefore concluded that the method is sufficiently accurate to allow for making comparative carbon dioxide output determinations on the animals used.

Indirect evidence that the factors discussed above are not important in producing error is obtained by comparing the results obtained on different animals. Errors caused by the passing through of excess carbon dioxide would tend to increase the apparent result for small animals more than for larger ones, while for small animals the error resulting from the loss of carbon dioxide that was not absorbed would tend to be minimized. With these ideas in mind, if one looks at the results for *Rana palustris* (Table I.) it is apparent that Nos. 6–17 have within the limits of variation the same carbon dioxide output result as No. 5, although the latter has twelve times the chance of being thrown off by the errors as have the former. Again *Bufo americanus* shows only slightly higher results for a 1.9 gm. individual as for an 11 gram one, and *Rana clamitans* shows a lower result for the lighter animal.

V. Complicating Factors.

A. Cell Size.

1. If the figures presented in this paper are compared with those obtained by other workers, it will be found that they run decidedly low. Measurements are presented for the same species only in the case of *Amphiuma* and *Cryptobranchus*, but the indications are in the same direction for the frogs and toads, in which different species have been used here than those used by other workers. There are two possible causes for this difference; first that the writer has obtained shrinkage of the corpuscles, as previously pointed out, and second that the measuring technique used is faulty. Several attempts have been made to check the latter point. The scale used has been repeatedly compared with a Zeiss stage micrometer, and found to be accurate. By means of an ocular filar micrometer the widths of the different 10 micron divisions of the Zeiss stage micrometer have been measured.

TABLE IV.

VARIATION IN RED BLOOD CELL SIZE IN DIFFERENT SPECIES OF AMPHIBIA.

Number of corpuscles measured corresponding to each size.

Size Micra.	*Amph.*		*Nec.*		*Crypt.*		Size Micra.	*Diem.*		*R. Cat.*		*R. Clam.*		*R. pip.*		*R. pal.*		*Hyla.*		*Acris.*		*Coro.*		*Bufc.*	
	L.	W.	L.	W.	L.	W.		L.	W.	L.	W.	L.	W.	L.	W.	L.	W.	L.	W.	L.	W.	L.	W.	L.	W.
83	1						34			1															
77	2						33	1		3															
76	1						32	7		6															
74	1						31	11		16															
73	2						30	10		38															
72	3						29	9		109		3													
71	6						28	5		120		5													
70	5		2				27	4		149		22		4											
69	7						26	3		230		40		4		1									
68	8		5				25	3		214		77		30		4									
67	18		7				24			97		140		82		16		1							
66	17		7				23			62		155		111		57									
65	24		15				22			37		121		150		98		3							
64	14		12				21			14	1	104		143		142		8							
63	37		16				20		5	7	1	86		162		305		8		7		7			
62	32		33				19		10	1	7	45		122		202		18		18		12		3	
61	27		34				18		16		33	24		63		128		10		37		14		37	
60	32		62				17		10		51	8		16	3	51		2		22		9		53	
59	19		52				16		5		46	1	2	4	13	6	1			14		5		67	
58	21		85				15		3		148		29		132		21			2		3		65	
57	13		99				14		1		292		105		296		112							18	
56	15		98				13				314		186		233		323		3		3			6	
55	16		139				12				130		231		124		379		15		28		2	1	1
54	7		107				11				44		148		58		144		22		32		8		30
53	3		111				10				24		78		33		20		9		30		31		121
52	2		167				9				2		1		11				1		8		8		85
51	5		122				8																1		13

TABLE IV.—*Continued.*

Size Micra.	*Amph.*		*Nec.*		*Crypt.*		Size Micra.	*Diem.*		*R. Cat.*		*R. Clam.*		*R. pip.*		*R. pal.*		*Hyla.*		*Acris.*		*Coro.*		*Bufo.*	
	L.	W.	L.	W.	L.	W.		L.	W.	L.	W.	L.	W.	L.	W.	L.	W.	L.	W.	L.	W.	L.	W.	L.	W.
50	5		164																						
49			95																						
48	1		74																						
47			74																						
46			49		1																				
45	1	1	31		4																				
44	1	5	9		3																				
43		4	9		8																				
42		7	2		8																				
41		13	1		5																				
40		28	1	1	7																				
39		21	1	6	4																				
38		37		9	2																				
37		31		21	3																				
36		52		33	3																				
35		47		57	1																				
34		28		61																					
33		28		110		1																			
32		20		131																					
31		14		151																					
30		7		176																					
29		4		161																					
28		2		149																					
27				138																					
26				123		3																			
25				138		3																			
24				95		10																			
23				63		8																			
22				36		10																			
21				20		7																			
20				6		3																			
19				1		1																			
Totals	350		1700		50		Totals	50		1100		830		900		1000		50		100		50		250	

It is found that these divisions vary in width from 5.8 per cent. below the mean to 5.6 per cent. above it. With such wide variations between the different divisions of the micrometer, the question naturally arises as to whether the entire micrometer may not be inaccurate. As a third check on the method the dimensions of corpuscles as measured by means of the scale were compared with the dimensions of the same corpuscles as measured by means of the filar micrometer. It was found that the scale is less accurate for individual corpuscles, because it is impossible to measure with it to an accuracy of less than one micron, but the average of a series of measurements by the two methods gave closely parallel results.

2. In the Amphibia the size of individual red blood cells in the same animal varies so widely that averages only partly represent the peculiarities of the different species. In many cases

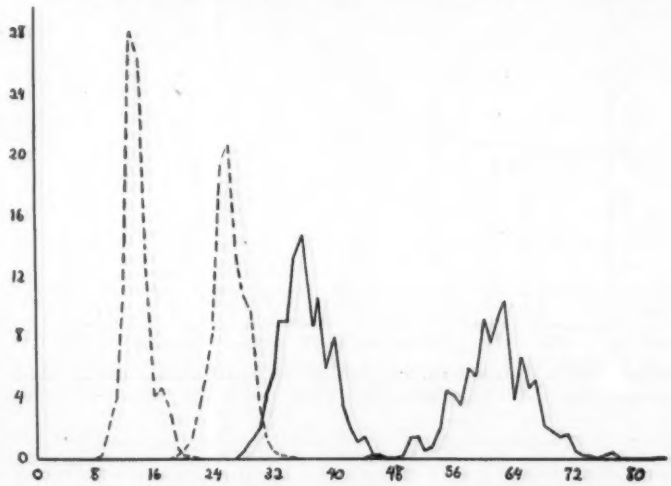

CHART 1a. Variation in red blood cell size for *Amphiuma* (solid line) and *Rana catesbiana* (broken line). Abscissa, dimensions in micra; ordinate, percentage of total corpuscles measured. Two curves are given for each species, one representing the long diameters of corpuscles, and the other the short diameters.

the range of variation in size is just as characteristic as the average size. For this reason Table IV. has been prepared to show the range of size variation in each species. In many cases

there are either two maxima or a sustained maximum. This results from the fact that the maximum for different individuals of the same species varies. It is just this variation between individuals which complicates comparative results on cell size, especially between closely related species. It is not associated with the metabolic activity of the animal, sex, or any other factor which can at present be indicated. Reference to Chart I will aid in understanding the variations here discussed.

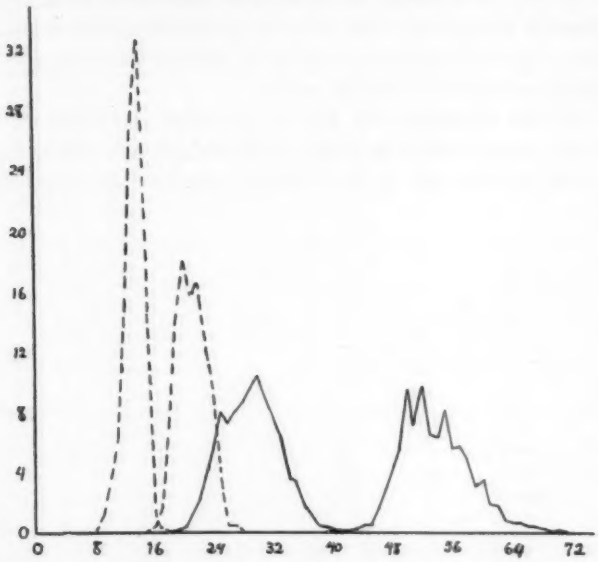

CHART 1*b*. Variation in red blood cell size for *Necturus* (solid line) and *Rana pipiens* (broken line). Explanation as for Chart 1*a*.

B. Carbon Dioxide Production.

1. The weight of the animal is the primary factor which complicates the results on carbon dioxide production. The general fact is that the carbon dioxide output as measured by unit weight increases as the weight decreases. Reference to the results for *Necturus* will emphasize this fact. For convenience these are listed in Table V., the individuals being taken in order of their weight.

TABLE V.

Necturi ARRANGED ACCORDING TO WEIGHT TO SHOW VARIATION OF CARBON DIOXIDE PRODUCTION WITH VARIATION IN WEIGHT.

No.	Weight, Grams.	CO_2, Mg.	No.	Weight, Grams.	CO_2, Mg.	No.	Weight, Grams.	CO_2, Mg.
3	194	0.0413	6	115	0.0532	17	64	0.1192
18	194	0.0599	13	93.5	0.0842	8	57.6	0.0922
2	154	0.0420	14	92	0.0753	9	55.7	0.1069
5	130	0.0548	10	86	0.0883	11	47	0.1399
4	126	0.0528	12	75	0.0915	16	44.3	0.1208
7	126	0.0557	15	74	0.1051			

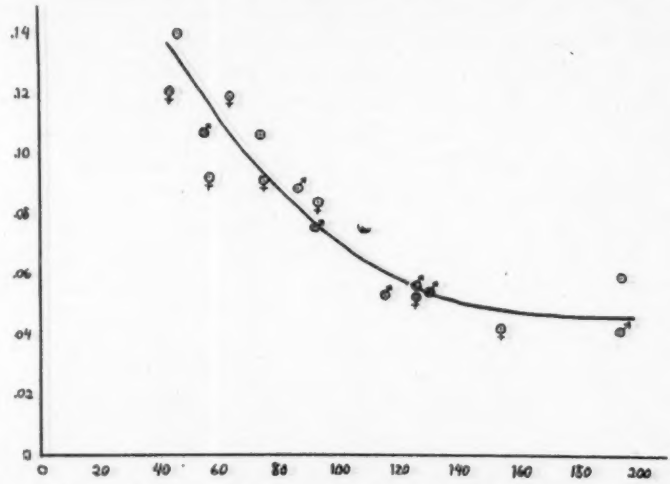

CHART 2. Influence of weight on carbon dioxide output in *Necturus*. Abscissa. weight in grams; ordinate, CO_2 in milligrams per gram of body weight per hour, ♀ = females, ♂ = males, ⊙ = sex not determined.

The general trend is evident from Table V., but exceptions are also evident. Nos. 18, 13, 15, 17, and 11 are higher than would be expected from their weight, while Nos. 2, 6, 14, and 8 are lower. The high group contains only animals on which one or two carbon dioxide output tests could be made before the animal died. The cause of death in all these animals was probably starvation, there being no pathological symptoms as far as could be determined. A more detailed discussion of starvation as a complicating factor will be found below. It is more difficult to

understand the meaning of the abnormally low carbon dioxide production. All the individuals in the low group except No. 14 were used for a number of trials; 14 died after 105 days of starvation when only one trial had been made. Nos. 2 and 8 are females; 6 and 14 males. Females usually run lower than males (see below) but this explains only two of the cases. The detailed record of No. 6 shows a low initial period, a fluctuating intermediate period, and a very high final one as starvation progressed. The final period was characterized by a rapid loss of weight.

In the other species studied the same inverse ratio between body weight and metabolism is evident. In *Amphiuma* the result is strikingly higher for the smallest animal, and unusually constant for the three larger specimens. In the frogs the same trend may be observed, but it is not as much in evidence, probably because of the preponderance of other complicating factors.

These observations suggest that weight is not a thoroughly satisfactory basis for computing basal metabolism in these animals. The ideal basis on which to make such computations would be mass of respiring tissue in the body. In Amphibia this mass would be less in proportion to the total body weight in *Salientia* than in *Caudata*, due to the greater mass of bone in the former. This proportion would also be smaller in large animals than in small individuals of the same species, because of the increased ossification and connective tissue in the former. In man it has been found that the body surface is the more reliable criterion, and elaborate formulæ have been worked out for computing this surface from the weight and height. In Amphibia such formulæ would be useless, on account of the great variation in shape which is found between the various species. It appears that the most hopeful method of eliminating weight variations in comparing different species as to carbon dioxide output is to choose animals from each species which have approximately equal weights. In the final section of this paper an attempt is made to do this.

2. Starvation is a factor leading to important variations in the results, especially with *Necturus*. The rate of carbon dioxide elimination increases as starvation progresses. This fact has already been indicated by the observations on Necturi with

abnormally high carbon dioxide output. Table VI. and Chart 3 illustrate this variation. The two specimens chosen were both starved for a long period, and determinations were made throughout the period.

Both these animals show unmistakably the upward trend of the production of carbon dioxide with increased length of starvation, even beyond that which can be accounted for on the basis of decreasing weight during starvation. The writer was led to make the computations on the basis of standard weight because a preliminary examination of the results had suggested that the actual increase observed was largely or entirely due to this factor. These results mean, then, that the reduced amount of living tissue resulting from starvation actually produces a greater absolute amount of carbon dioxide than the greater amount of living tissue present at the beginning of starvation.

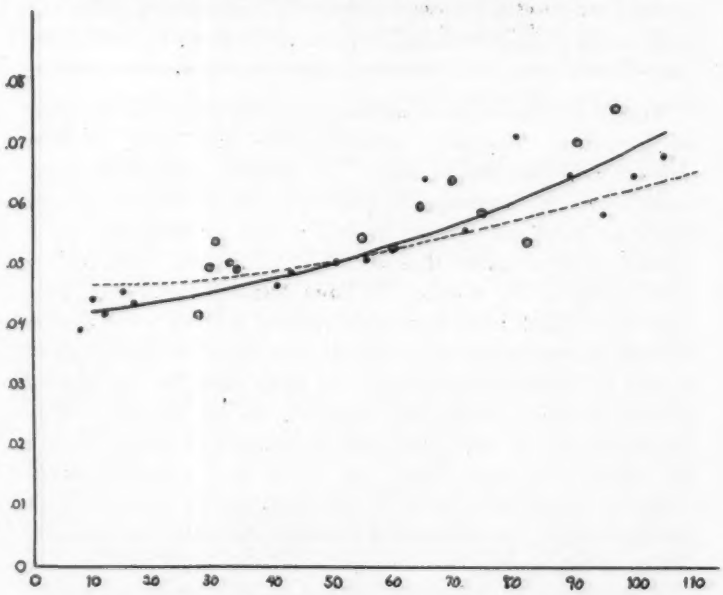

CHART 3. Influence of starvation on carbon dioxide output in *Necturus*. Abscissa, number of days starved; ordinate, CO_2 in milligrams per gram of body weight per hour. Dots refer to *Necturus* No. 4; circles to *Necturus* No. 7. The broken curve is based on the "standard weight" figures in Table VI.

TABLE VI.

EFFECT OF STARVATION ON CARBON DIOXIDE OUTPUT OF *Necturus*.

Necturus 4.				*Necturus* 7.			
Days Starved.	Weight, Gm.	CO_2, Mg.	CO_2 Based on Standard Weight.	Days Starved.	Weight, Gm.	CO_2, Mg.	CO_2 Based on Standard Weight.
8	139	0.0381	0.0420	27	140	0.0417	0.0463
10	139	0.0439	0.0484	29	136	0.0496	0.0535
12	139	0.0422	0.0465	31	136	0.0539	0.0582
15	138	0.0451	0.0498	33	136	0.0502	0.0542
17	138	0.0435	0.0480	34	136	0.0485	0.0522
41	129	0.0466	0.0477	55	127	0.0546	0.0550
46	127	0 0485	0.0489	60	127	0.0527	0.0531
51	127	0.0509	0.0513	64	121	0.0596	0.0572
56	123	0.0511	0.0499	70	121	0.0636	0.0611
66	121	0.0640	0.0615	75	121	0.0586	0.0562
72	120	0.0559	0.0532	82	115	0.0536	0.0489
81	118	0.0713	0.0668	91	113	0.0702	0.0630
89	117	0.0647	0.0601	97	109	0.0756	0.0652
95	117	0.0579	0.0538				
100	113	0.0646	0.0579				
104	112	0.0678	0.0603				

Results on other species are less conclusive regarding the effect of starvation. Extensive determinations were made on *Rana pipiens* with this idea in mind. No changes were noted which could be directly attributed to starvation, except that the extreme inanition previously noted in No. 2 was accompanied by a decided drop in carbon dioxide output at the end. No *Necturi* were carried as far as this. In *Rana palustris* the animals used when near death from starvation showed a much lower carbon dioxide output value than normal starving individuals (about 0.1660 as compared to 0.2730). In *Rana clamitans* the starved individual gave a lower result than the one less starved. With *Amphiuma* No. 2, the carbon dioxide elimination decreased with the weight. It seems, then, that in all species studied except *Necturus* starvation resulted in decreased carbon dioxide output per unit weight, but evidence is offered to show that the opposite is true of this species.

3. Reference to Table VI. will show that there are rather large daily fluctuations in the carbon dioxide output of *Necturus*. Daily records for individuals of other species show the same sort of variation. The possibility is not eliminated that these fluctua-

tions are the result of differences in the aëration rate. This factor could not be accurately controlled by the apparatus used. However careful observation and comparison of the results of over 250 aërations has convinced the writer that the variations noted are not the result of differences in aëration rate. Many results were discarded in which there was good reason to believe that an abnormally slow aëration had produced a low result. Daily temperature records of the room in which the experiments were carried on were made, and these records show no fluctuations which could possibly account for the daily variations found in the results for individual animals. It is tentatively concluded that we are dealing with unexplained variations in the metabolism of the animal.

4. Sex is responsible for some minor variations in the results. It is known that basal metabolism proceeds at a higher rate in men than in women. The same appears also to be true of Amphibia. If comparable weights be chosen, it is found that *Necturi* female No. 4 is lower than male No. 7, and that female No. 8 is lower than male No. 9. *Rana palustris* females Nos. 2 and 4 are lower than male No. 3.

5. Disease was responsible for some aberrant results in *Rana pipiens*. Several of the animals (Nos. 1, 3, 7 and 8) died of a disease which the writer has called "lymph œdema." It is accompanied by an accumulation of lymph, or water, in the subcutaneous lymph sinuses, leading to a marked increase in weight and a swollen appearance. In the later stages this was invariably accompanied by capillary bursting in the skin and muscles. On autopsy the liver was spotted and the spleen enlarged and crowded with blood. All the animals that died from such œdema showed a sharp rise in carbon dioxide production at the onset of the symptoms. This production remained high until death in spite of the increased weight which would tend to reduce the carbon dioxide per gram weight. For instance No. 3 had a rise from 0.1521 to 0.2427 at the onset of the disease, and died with a production of 0.1798 mg. per gm. of weight in spite of a 25 per cent. increase in weight. No. 6 died from a complication of causes, which included a brain tumor connected with the posterior choroid plexus on the right side, a fatty degeneration which involved the entire right kidney and part of the left,

fatty degeneration of the spleen, lungs and body cavity filled with water, partial paralysis shown by inability to draw up rear legs or to support the body with the right fore leg, and a twisting of the head to the right. This animal showed a fall in carbon dioxide production from 0.1880 down to 0.1239 mg. per gm. per hour, and retained approximately the latter rate until death, in spite of a progressive increase in the severity of the symptoms. Other species did not yield much opportunity to study the effects of disease on metabolism. *Necturus* No. 12 died of fish mould. The determination made on this animal while normal yielded 0.0915 mg. per gm. per hour, while a determination made during the active progress of the infection yielded 0.1354 mg.

6. Motility of the experimental animal may be an important cause of variation in the results obtained. All the animals used were given an hour to get accustomed to the jar before each determination, and there was very little movement in the majority of cases. Salientians would shift their position occasionally, but did very little struggling. A few individuals struggled considerably during the first test made on them. The results of such experiments were discarded. It is interesting that the struggling resulted in approximately doubling the basal rate of carbon dioxide production. *Caudata* struggled very little or not at all.

A few trials were made using curare, which paralyzes the muscles, to see whether more constant results could not be obtained. It was found that the carbon dioxide production of *Rana pipiens* is thus reduced about 25 to 35 per cent., but the daily variations persist. The method described by Lund (1919) of placing the respiring animal in the jar with the barium hydroxide (suspended from the stopper in a basket) was tried on curarized animals. The results checked fairly well with those obtained on the same animals by the aëration method, but it was found that a considerable error is introduced by the necessity of removing the animal from the jar at the end of a measured time; a procedure which stirs up the air in the jar and causes loss of carbon dioxide.

VI. General Comparative Results.

A. Comparison of Classes of Vertebrata.

Table VII. has been prepared to show the cell size variations between the various classes of vertebrates, as indicated by measurements made on corpuscles from a selected representative of each class.

Table VII.

Red Blood Cell Size in Selected Species of Vertebrata.

Class.	Species.	Red Blood Corpuscles.		
		Length.	Width.	Area.
Amphibia.....	*Rana pipiens*	20.494	13.153	422
Reptilia.....	*Crotalus adamanteus*	19.0	11.0	349
Pisces.....	*Ambloplites rupestris*	12.37	8.13	158
Aves.....	*Gallus domesticus*	12.3	6.7	130
Mammalia.....	*Homo sapiens*	7.9 [1]	7.9	98

The frog is below the average of red blood cell size for Amphibia, and the rock bass is below the average for fish. Man is above the average for mammals. The chicken and rattlesnake are near the average for their respective classes. The arrangement of the classes in order of increasing activity would result in the same order as that in Table VII. The avian corpuscle is nucleated, while that of the human is not. There is less difference in bulk of hemoglobin between the two than the measurements would indicate.

B. Comparison of Different Species of Amphibia.

In Table VIII. the species used in this study are listed in order of their red blood cell size, and columns are filled in for weight and carbon dioxide production. All the figures are averages of the detailed results recorded in Table I.

A study of Table VIII. shows that the general trend is clearly in the direction of increasing carbon dioxide output with decreasing cell size, but the results are complicated by the fact that the species with large cells are also large in size. To eliminate the weight factor, representatives of several species have been chosen which have comparable weights, and the results from these

[1] Taken from Gulliver (1875).

TABLE VIII.

AVERAGES OF RED BLOOD CELL MEASUREMENTS AND CARBON DIOXIDE OUTPUT BY AMPHIBIA.

Species.	Weight, Grams.	CO_2 Mg. per Gm. per Hr.	Red Blood Corpuscles.		
			Length.	Width.	Area.
Amphiuma means	1,013.6	0.0390	61.79	36.696	3,561
Necturus maculosus	101.6	0.0814	53.416	28.617	2,401
Cryptobranchus alleghenienſis			41.12	23.16	1,496
Diemyctylus viridescens			29.64	17.76	827
Rana catesbiana	511	0.07001	25.66	14.184	572
Rana clamitans	14	0.1576	22.98	11.916	429
Rana pipiens	38	0.1632	20.494	13.153	422
Rana palustris	3.3	0.1820	20.379	12.683	406
Hyla pickeringii			19.44	11.2	342
Acris gryllus			17.76	10.88	304
Chorophilus nigritus			17.96	10.04	283
Bufo americanus	6.4	0.1950	16.004	9.684	244

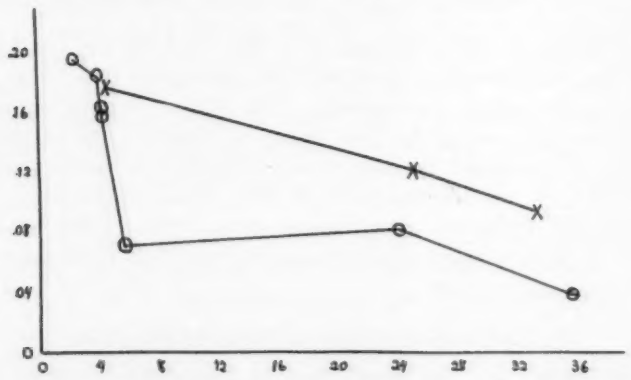

CHART 4. Cell size and carbon dioxide output in Amphibia. Abscissa, area of red blood cells in hundreds of square micra; ordinate, CO_2 in milligrams per gram of weight per hour. Curve based on circles represents the averages taken from Table VIII.; curve bases on *X* represents the individuals recorded in Table IX.

individuals have been recorded separately in Table IX. On Chart 4 are plotted two curves, one based on Table VIII., showing the general upward trend of carbon dioxide output with decreasing cell size, but obviously complicated by the weight factor; the other based on Table IX., showing the even curve which is obtained when weight variations are eliminated.

TABLE IX.

RED BLOOD CELL SIZE AND CARBON DIOXIDE PRODUCTION OF INDIVIDUALS WITH COMPARABLE WEIGHT.

Species and No.	Weight, Grams.	CO_2, Mg.	Red Blood Corpuscles.		
			Length.	Width.	Area.
Amphiuma means, 2	41.4	0.0920	60.9	33.8	3,329
Necturus, 16	44.3	0.1208	50.38	31.66	2,505
Rana pipiens, 3	45.8	0.1768	21.08	14.19	470

The results recorded in Tables VIII. and IX. and in Chart 4 furnish conclusive evidence that there is a correlation between the degree of activity of a species as determined by its carbon dioxide output and the size of its red blood cells. The physiological necessity for such a correlation lies in the necessity for having sufficient surface to allow for the exchanges which take place between surface and interior. If the exchange is rapid, the surface must be large, and this enlargement of surface is brought about by having the mass divided into smaller packets.

VII. SUMMARY AND CONCLUSIONS.

1. Cell size has been measured in a number of species of Amphibia by measuring the dimensions and computing the area of red blood corpuscles. Metabolic activity was measured by collecting in barium hydroxide the carbon dioxide produced by the animal, and computing the carbon dioxide in milligrams per gram of body weight per hour.

2. Comparison of the measurements of red blood corpuscles with measurements published by other authors indicates that the measurements obtained by the present writer are too low. The cause of these low results is unknown.

3. Controls on the method used in determining carbon dioxide output indicate that this method was not extremely accurate, but sufficiently so to allow for making comparisons between the animals used. The chief sources of error were loss of carbon dioxide due to incomplete absorption, and inability to maintain a constant rate of air flow. Indirect evidence that the method is approximately correct is obtained by comparing actual results.

4. There is shown to be a size variation between the corpuscles

of the same individual, and a variation in the average size from different individuals of the same species.

5. A number of factors tend to complicate the results on carbon dioxide production. The most important is body weight, the smaller animals producing more carbon dioxide per unit of weight than the larger animals. This factor becomes especially important in making comparisons of different species, because species vary quite widely in their average weight.

Starvation in *Necturus* seems to cause an increase in the carbon dioxide output per unit of weight, even when all results on an individual are reduced to a constant weight value. In the other species studied starvation seems to lead to a decrease in carbon dioxide production.

Other factors complicating carbon dioxide output results are daily variations in the metabolism of the individual; sex, the male producing slightly more than the female; disease, usually resulting in an increased production, a fact which suggests the fever response in man; and the movement of the animal. This last factor may become of extreme importance if the animal is active, but an attempt has been made to eliminate such results by keeping the animals quiet and discarding results of determinations made on actively moving animals.

6. The conclusion that cell size varies inversely with metabolic activity is justified by the evidence presented. This is shown in a general way by comparing classes of Vertebrata, and more specifically by detailed results on twelve species of Amphibia.

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